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Ultra high-pressure liquid chromatographic assay of moxifloxacin in rabbit aqueous humor after topical instillation of moxifloxacin nanoparticles

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ABSTRACT

The present report describes a rapid and sensitive ultra high-pressure liquid chromatography (UHPLC) method with UV detection to quantify moxifloxacin in rabbit aqueous humor. After deproteinisation with acetonitrile, gradient separation of moxifloxacin was achieved on a Waters Acquity BEH C18 (50 mm × 2.1 mm, 1.7 μm) column at 50 °C. The mobile phase consisted of 0.1% trifluoroacetic acid in water and acetonitrile at a flow rate of 0.4 ml/min. Detection of moxifloxacin was done at 296 nm. Method was found to be selective, linear (r = 0.9997), accurate (recovery, 97.30–99.99%) and precise (RSD, ≤1.72%) in the selected concentration range of 10–1000 ng/ml. Detection and quantitation limit of moxifloxacin in aqueous humor were 0.75 and 2.5 ng/ml, respectively. The aqueous humor levels of moxifloxacin after single topical instillation in three formulations, i.e. moxifloxacin solution (Moxi-SOL), anionic nanoparticles (Moxi-ANP) and cationic nanoparticles (Moxi-CNP) were investigated. A fourfold increase in the relative bioavailability was observed with the Moxi-CNP ($AUC_{0\rightarrow t}$, 3.14 μg h/ml) compared with Moxi-SOL ($AUC_{0\rightarrow t}$, 0.79 μg h/ml) and Moxi-ANP ($AUC_{0\rightarrow t}$, 0.72 μg h/ml) formulation. The results indicate that the cationic nanoparticle increases ocular bioavailability of moxifloxacin and prolong its residence time in the eye.

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1. Introduction

Moxifloxacin, a fourth generation fluoroquinolone, is a broad-spectrum antibiotic used in the prevention and treatment of a variety of ocular infections [1]. Recent reports based on several in vivo studies have shown the potency of moxifloxacin in preventing anterior eye infections such as bacterial conjunctivitis and keratitis [2]. Traditionally the plasma concentration of moxifloxacin and its relation to the minimum inhibitory concentration has been used to predict its likely efficacy against ocular infections and therefore most of the bioanalytical methods currently available for the quantification of moxifloxacin are restricted to measuring plasma or serum concentration of moxifloxacin in pharmacokinetic studies [3–16]. For extravascular infections such as ocular infections, the ability of antibiotic to kill and eradicate the pathogens at the site of infection (aqueous humor) is an important goal and therefore determination of drug concentration in aqueous humor is desirable and is needed. As for moxifloxacin concentration in eye, the only assay described in the literature was based on HPLC with amino acid HPLC column and concerned the measurement of moxifloxacin using fluorescent detection [17]. However, the chromatographic run time of the method was long with retention time of moxifloxacin at 16.7 min [17]. In this paper, the UHPLC method with UV detection for determination of moxifloxacin in rabbit aqueous humor is described. We propose the procedure with minimal sample pre-treatment using direct injection into chromatographic column. Furthermore, the rabbit aqueous humor concentrations of moxifloxacin solution (Moxi-SOL), its negatively charged nanoparticles (Moxi-ANP) and its positively charged nanoparticles (Moxi-CNP) following topical administration were evaluated.

2. Experimental

2.1. Chemicals

Gift sample of moxifloxacin was provided by Ranbaxy Laboratories Ltd. (Gurgaon, Haryana, India). Acetonitrile of HPLC grade was obtained from Qualigens Fine Chemicals (Mumbai, India) and water was produced in the laboratory by a Milli-Q purification sys-

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Table 1
Gradient elution programme for the analysis of moxifloxacin.

S. no.	Time (min)	Flow rate ml/min	A (%)	B (%)	Curve
1	Initial	0.40	80.0	20.0	6
2	0.50	0.40	80.0	20.0	6
3	1.00	0.40	70.0	30.0	6
4	2.00	0.40	70.0	30.0	6
5	3.00	0.40	80.0	20.0	6
6	4.00	0.40	80.0	20.0	6

A: 0.1% TFA in water; B: acetonitrile.

tem (Millipore, Billerica, MA, USA). Trifluoroacetic acid (TFA) of Chrom Lichro grade was purchased from Merck Ltd. (Worli, Mumbai, India). All other reagents used were of analytical grade.

2.2. Chromatography

UHPLC analysis was performed on a Waters Acquity UHPLC system (Milford, MA, USA) equipped with a binary solvent manager, an auto sampler, column manager composed of a column oven, a pre-column heater and a photo diode array detector. Five microliter of final analytical solution was injected into a Waters Acquity BEH C18 (50 mm × 2.1 mm, 1.7 μm) UHPLC column kept at 50 °C and the chromatographic separation was performed by gradient elution (Table 1). The mobile phase consisting of a mixture of A: 0.1% TFA in water (pH 3.5) and B: acetonitrile, with the flow rate of 0.4 ml/min was employed. The analysis was performed at 296 nm wavelength with total run time of 4 min. Data acquisition, data handling and instrument control were performed by Empower Software v1.0.

2.3. Sample preparation

A 50 μl aliquot of rabbit aqueous humor was pipetted into a 1.0 ml Eppendorf tube and 100 μl of acetonitrile was added. The samples were vortex mixed for 2 min followed by filtration through 0.22 μm nylon filter and 5 μl of filtrate was directly injected into the UHPLC system. All the rabbit aqueous humor samples were stored at −20 °C and were allowed to thaw at room temperature prior to sample preparation.

2.4. 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 prepared daily by dilution of stock solution with acetonitrile. To prepare the aqueous humor calibration standards, aliquots of 50 μl of aqueous humor were placed in each Eppendorf tube and spiked with increasing concentrations of working standard solutions to give moxifloxacin concentrations of 10, 20, 100, 200, 400, 600, 800 and 1000 ng/ml. Calibration standards were processed according to sample preparation procedure and were analyzed by UHPLC method.

2.5. Validation and stability

The UHPLC method was validated in terms of linearity, specificity, sensitivity, precision, accuracy, system suitability and robustness [18]. The stability of moxifloxacin in aqueous humor at 4 and 20 °C and after freeze–thaw cycles was also determined.

2.6. Preparation of moxifloxacin nanoparticles

For the preparation of cationic nanoparticles (Moxi-CNP) the double emulsification solvent diffusion method was used [19]. Briefly, chitosan (0.1%, w/v) was dissolved in 50 ml of acetic acid buffer, pH 4.4, which also contained polyvinyl alcohol (1%, w/v). Primary w/o emulsion formed by dropwise addition and subsequent stirring of 1 ml of aqueous solution of moxifloxacin (1.0%, w/v) into 10 ml of dichloromethane containing poly (lactide-co-glycolide) (1.0%, w/v) was poured into the chitosan aqueous solution. The emulsion was stirred at 1500 rpm till complete evaporation of dichloromethane. The nanosuspension so formed is passed through three cycles of homogenization at 10,000 bar pressure. After particle formation the entire dispersed system was centrifuged (10,000 rpm, 15 min) and the sediment was resuspended in Milli-Q water. This process was repeated and the resultant dispersion was subjected to freeze–drying. Anionic Moxi-ANP particles (with no chitosan) were also prepared for comparison. The mean hydrodynamic diameter and zeta potential of Moxi-ANP and Moxi-CNP, as measured using Malvern Zetasizer Nano-ZS90, were found to be 110.5 nm (−23.5 mV) and 120.7 nm (+32.5 mV), respectively.

2.7. Ocular pharmacokinetic study

Three groups, each having seven New Zealand Albino rabbits (2.25 ± 0.25 kg), were used for the ocular study. The protocol was approved by Institutional Animal Ethics Committee, Jamia Hamdard (approval no. 469) and their guidelines were followed. For topical instillation, weighed amount of lyophilized Moxi-ANP and Moxi-CNP was dispersed in isotonic buffer (pH 7.2) to form 0.5% moxifloxacin suspension. Moxi-SOL (0.5%) was also prepared in the same vehicle. Each group received single topical instillation of Moxi-SOL, Moxi-ANP and Moxi-CNP. The formulations were instilled in both the eyes and approximately 50 μl of aqueous humor was collected before instillation of formulations and post treatment at 0.5, 1, 2, 4, 6 and 12 h. Aqueous humor was collected from one rabbit (both eyes) at each time point. All aqueous humor samples were collected in pre-labeled eppendorf tubes, sealed and stored at −20 °C until UHPLC analysis. The aqueous humor samples were prepared as above mentioned.

3. Results and discussion

3.1. Method development

Moxifloxacin exists in solution as cationic, anionic, zwitterionic and neutral forms owing to the presence of two protonation sites, carboxyl and secondary amino piperazinyl group [7]. These neutral and ionic forms of moxifloxacin have significant difference in their apparent hydrophobicity and thus tend to migrate through column with different velocities resulting in poor peak shape, tailing and decreased sensitivity during HPLC method development [20]. The HPLC methods described previously for plasma pharmacokinetics of moxifloxacin were based on either pre-column derivatization or on-column focusing or fluorescence detection in order to achieve column efficiency, selectivity and sensitivity. The present study attempts to improve the analyte retention and chromatographic selectivity, using smaller Bridged Ethyl Hybrid column packed with small sized (1.7 μm) particles employing ultra high pressures. The small sized particles reduce plate height and consequently allow the number of theoretical plates to be increased. They also favour faster linear velocities. The use of smaller particles therefore allows reduction of analysis time and improved peak shape. Protein precipitation with acetonitrile and direct injection into chromatographic column enables high recovery of moxifloxacin. Gradient elution with varying compositions of TFA and acetonitrile was tried to improve the chromatographic separations. TFA acts as acidic modifier and provides counter ions which affect the moxifloxacin solvation. The ionic retention of protonated moxifloxacin with oppositely charged species results in formation of stable ion pairs which improves retention of moxifloxacin. The theoretical plates, USP tailing factor and retention time obtained for mox-

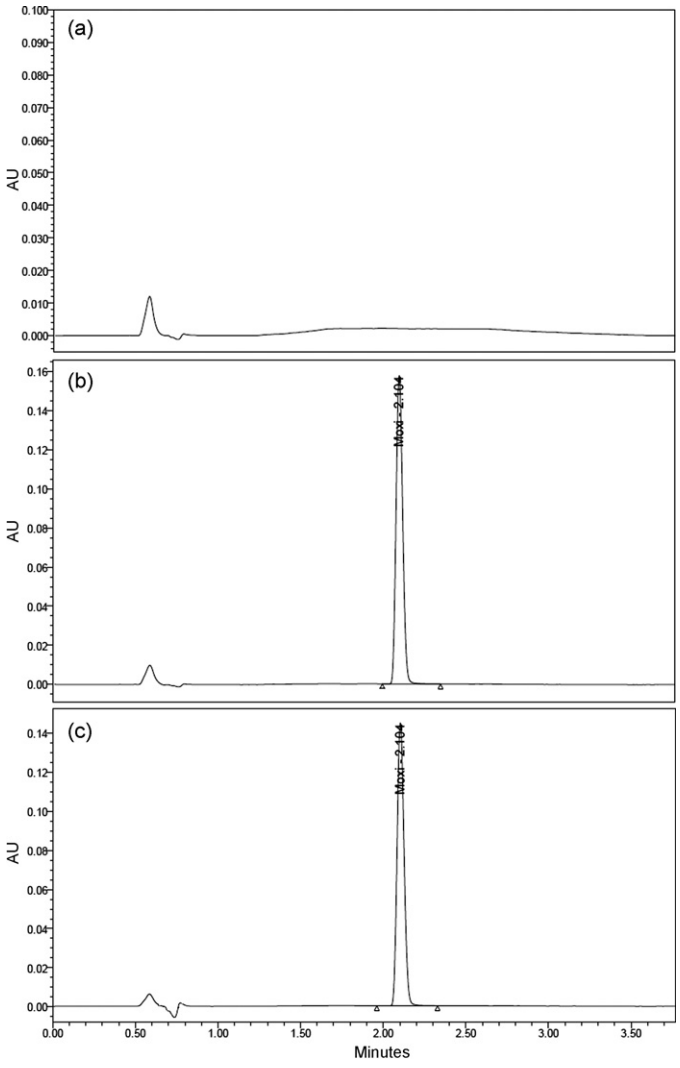

Fig. 1. UHPLC chromatograms obtained from (a) blank aqueous humor, (b) aqueous humor spiked with moxifloxacin (400 ng/ml) and (c) a rabbit's aqueous humor, 1 h after topical instillation of Moxi-CNP; moxifloxacin R_t = 2.10 min.

ifloxacin in aqueous humor matrix showed improvement when column was kept at a temperature of 50 °C.

3.2. Method validation

3.2.1. Linearity

The linearity of the detector response for the moxifloxacin was evaluated by injecting a total of eight calibration (working) standard solutions (10–1000 ng/ml) covering the working range of the assay. The calibration curves were constructed by plotting peak area of moxifloxacin against corresponding concentrations. The correlation coefficient for the calibration regression line was 0.9997 whereas the equation of calibration curve was $y = (903.29 \pm 3.42)x + (7200.7 \pm 17.2)$ where, x is the concentration of moxifloxacin in aqueous humor, and y is the peak area of moxifloxacin. Standard errors for slope and intercept were 1.4 and 7.0%, respectively.

3.2.2. Selectivity

Selectivity was demonstrated by the ability to assess unequivocally the analyte in the presence of endogenous matrix constituents. UHPLC chromatograms of blank aqueous humor (Fig. 1a), aqueous humor spiked with moxifloxacin (Fig. 1b) and a rabbit's aqueous humor after topical instillation of Moxi-CNP (Fig. 1c) were compared to show the selectivity of the proposed procedure. The retention time of moxifloxacin was 2.10 min and no interference was observed either by matrix or by formulation ingredient, near the retention time, demonstrating method's selectivity (Fig. 1).

Table 2
Precision and accuracy of UHPLC method.

Nominal amount (ng/ml)	Amount found (ng/ml)	Precision			Accuracy (%)
		SD	SE	RSD (%)	
Intra-day					
10	9.73	0.09	0.05	0.92	97.30
100	98.57	1.08	0.62	1.09	98.57
1000	998.29	0.68	0.39	0.07	99.83
Inter-day					
10	9.83	0.10	0.06	1.02	98.30
100	98.02	1.69	0.98	1.72	98.02
1000	999.86	1.66	0.96	0.17	99.99

3.2.3. Precision and accuracy

Precision and accuracy were performed by triplicate analysis of aqueous humor samples spiked with moxifloxacin at concentrations of 10, 100 and 1000 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 percentage relative standard deviation of measured concentrations for each calibration level, whereas accuracy was expressed as percent recovery [amount found/nominal amount × 100] of drug added to the blank aqueous humor. Table 2 summarizes the results of intra and inter-day precision and accuracy of the moxifloxacin assay.

3.2.4. Detection limits (DL) and quantitation limits (QL)

DL and QL were experimentally estimated by analysis of aqueous humor samples spiked with serially diluted moxifloxacin standard until the signal-to-noise ratio reached 3 and 10, respectively. DL and QL were found to be 0.75 ng/ml and 2.5 ng/ml, respectively. The present method has a 13-times higher sensitivity than that reported in previous assay for moxifloxacin determination in humor samples [17].

3.2.5. System suitability

System suitability was determined by six replicate injections at a concentration of 200 ng/ml. The results passed all the common USP acceptance criteria (Table 3).

3.2.6. Robustness

The low values of % RSD ($\leq$1.74) and SE (<1) obtained after introducing small deliberate changes in the developed UHPLC method indicated the robustness of the method.

3.3. Stability studies

Moxifloxacin was found to be stable in aqueous humor at 20 °C for at least 24 h and at 4 °C for 2 days with average recovery of 95.7 and 97.6%, respectively. The freeze–thaw data indicated that three cycles can be tolerated without losses greater than 10%. Determination of the stock solutions stability in mobile phase revealed no significant losses for at least 5 days at 20 °C.

3.4. Ocular pharmacokinetic study

The UHPLC method was successfully used to quantify moxifloxacin in aqueous humor samples collected following topical

Table 3
System suitability for moxifloxacin in aqueous humor.

Injection[a]	R_t (min)	Peak area	USP tailing
1	2.10	187,859	1.1
2	2.10	191,356	1.1
3	2.10	186,782	1.1
4	2.10	189,580	1.1
5	2.10	187,466	1.1
6	2.10	187,598	1.1
Mean	2.10	188,440	1.1
% RSD	0.0	0.9	0.0

[a] Replicate injections of 200 ng/ml moxifloxacin spiked aqueous humor.

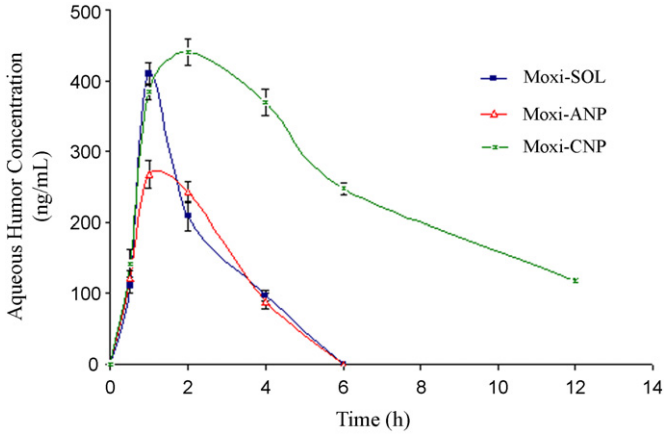

Fig. 2. Aqueous humor concentration–time profile of moxifloxacin after topical instillation of Moxi-SOL, Moxi-ANP and Moxi-CNP to rabbit eyes.

instillation of Moxi-SOL, Moxi-ANP and Moxi-CNP to rabbit eye. The resulting moxifloxacin concentrations measured in aqueous humor collected at 0.5, 1, 2, 4, 6 and 12 h are shown in Fig. 2. In the group treated with the Moxi-SOL and Moxi-ANP the aqueous humor levels of drug were undetectable after 4 h, attributed to their rapid precorneal loss. In contrast, drug was detected in aqueous humor for at least 12 h (214 ± 19.09 ng) following Moxi-CNP. A fourfold increase in the relative bioavailability was observed with the Moxi-CNP ($AUC_{0\rightarrow t}$, 3.14 μg h/ml) compared with Moxi-SOL ($AUC_{0\rightarrow t}$, 0.79 μg h/ml) and Moxi-ANP ($AUC_{0\rightarrow t}$, 0.72 μg h/ml) formulation. The results indicate that the positively charged Moxi-CNP interacts with negatively charged cornea and conjunctiva prolonging ocular residence time and thus maintaining prolonged transcorneal drug concentration gradient. It could be attributed to the presence of chitosan which has ability to enhance the paracellular drugs transport by opening intracellular tight junctions of cornea. The results demonstrate that positively charged nanoparticles increases the ocular bioavailability of moxifloxacin compared to moxifloxacin solution or negatively charged nanoparticles.

4. Conclusion

A novel UV-UHPLC method having high reproducibility and sensitivity for the determination of moxifloxacin in rabbit aqueous humor was developed in this study. The advantages of our method are the short analysis time (4 min), high sensitivity (QL: 2.5 ng/ml) and a simple sample extraction. Our laboratory is actually involved in development of nano-formulations for ocular delivery of anti-infectives. The established method provides a reliable bioanalytical methodology for moxifloxacin pharmacokinetics in rabbit aqueous humor.

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