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QUANTITATIVE DETERMINATION OF MOXIFLOXACIN IN RABBIT OCULAR FLUIDS BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

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Abstract

A rapid and sensitive high-performance thin-layer planar chromatographic (HPTLC) method for quantification of moxifloxacin (moxi) in rabbit aqueous and vitreous humor was developed using liquid-liquid extraction with methanol/ ethylenediaminetetraacetic acid (EDTA) aqueous solution (9:1, v/v). Moxi was separated on precoated silica gel 60F254 plates using mixture of methanol: dichloromethane (1:1, v/v) as mobile phase. Densitometric detection was done at 330 nm. Linear calibration curve in the range of 20-120 ng spot^{-1} showed correlation coefficient > 0.99 for both aqueous and vitreous humor. The precision, expressed as coefficient of variation, was in the range of 2.4 - 5.3% and accuracy, calculated as percentage recovery, was between 98.4 and 104.0%. The method was sensitive with quantification limit 3.5 ng. The extraction efficiency of moxi was 98.2 ± 2.6% in aqueous humor and 97.6 ± 3.4% in vitreous humor. The method was successfully applied to study and compare ocular fluid levels obtained after single instillation of moxi solution and moxi nanoparticle (Moxi-MNP) to rabbit eye. The Moxi-MNP increases ocular drug bioavailability and prolonged drug residence time in the eye. In conclusion, the method is useful for quantitative determination of moxi in ocular fluids.

Keywords: *Aqueous humor, High-performance thin-layer chromatography, Moxifloxacin, Nanoparticle, Vitreous humor*

INTRODUCTION

Moxifloxacin, (moxi) 1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-[(4*aS*,7*aS*)-octahydro-6H pyrrolo [3,4-b] pyridin-6-yl] 4-oxo-3 quinoline carboxylic acid is a fourth generation fluoroquinolone with a broad spectrum of antibacterial activity, encompassing gram-negative and gram-positive bacteria [1]. The use of moxi is of special interest in ophthalmology because it was proved to be effective in the treatment of bacterial conjunctivitis and keratitis and in prevention of endophthalmitis [2]. Studies in the ophthalmic literature support the advantages of moxi, citing, increased potency and lower resistance capability [2]. However, most of the topically applied drugs are washed off from the eye and less than 5% of administered drug enters the eye resulting in low ocular bioavailability [3]. To surmount the above mentioned obstacle, we prepared the moxi loaded mucoadhesive nanoparticles (Moxi-MNP) which could be administrated topically as eye drop for prolonged release of moxi. Since the drug is incorporated into polymer, its pharmacokinetics profiles may be completely altered [4]. Several bioanalytical methodologies including high-performance liquid chromatographic–tandem mass spectrometry (HPLC–MS/MS) [5], HPLC with fluorescence [6-12] or UV detection [13,14], spectrofluorimetry [15], square wave adsorptive

voltammetry [16] and capillary electrophoresis [17] have been developed for the measurement of moxi, but are mainly restricted to plasma matrices. Recently, an HPLC method with fluorescence detection is described for the quantification moxi in aqueous and vitreous humor, but the method has high retention time (16.7 min) of moxi [18].

Presence of metal ions such as Cu, Fe and Zn as a component of ocular fluids makes the analysis of moxi problematic, since chelation between metal ion and carbonyl and carboxyl groups of moxi is a common phenomenon resulting in drug binding and precipitation [19,20]. Under the stability indicating high-performance thin-layer chromatographic (HPTLC) condition for moxi, described previously by our group [21], the quantitation of moxi in ocular fluids is difficult leading to poor drug recovery and peak tailing effects. The present work comes up from the modification of the reported method with aim to develop a non-time consuming, simple and sensitive HPTLC method for determination of moxi in ocular fluids with a quantification limit sufficiently low to support pharmacokinetic studies. Several chelating agents (EDTA, citric acid, oxalic acid, sodium diethanol glycine) are tried to see their effects on drug recovery and peak symmetry. The proposed method is also applied to monitor ocular fluid pharmacokinetics following topical instillation of moxi solution and Moxi-MNP to rabbit eye.

METHODS

Chemical and Reagents

Moxi was received as a gift sample from Ranbaxy Laboratories Ltd. (Gurgaon, Haryana, India) and certified to contain 99.62% (w/w) on dried basis. Medium molecular weight chitosan (CS) (with a deacetylation degree >80%) was received as a gift sample from India Sea Foods (India). Poly (lactide-co-glycolide) (PLGA, RES 503H) having a monomer ratio (lactic acid/glycolic acid) of 50:50 was purchased from Boheringer Ingheleim (Germany). All other chemicals and reagents used were of analytical grade and were purchased from Merck Ltd. (Worli, Mumbai, India). For validation of the method, aqueous humor and vitreous humor was obtained from the healthy rabbits. All samples were frozen at – 20°C until analysis.

Chromatography

Chromatography was performed as described previously [22,23]. Briefly, precoated silica gel on aluminium plates 60F-254 (20 cm ×10 cm, 200µm thickness, E. Merck, Germany) were used after spraying with solution of 5% EDTA, pH adjusted to 9.0 with a 10% (m/v) NaOH solution. The plates were dried in a horizontal position for 30 min at 110°C in an oven. The samples were spotted in the form of bands of width 5 mm with Camag 100 microlitre syringe using a Linomat V (Camag, Muttenz, Switzerland) sample applicator. The equipment parameters include constant application rate of 150 nL s^{-1}, slit dimension of 5 mm × 0.45 mm and scanning speed of 20 mm s^{-1}. The linear ascending development carried out in 20 cm ×10 cm twin through glass chamber (Camag, Muttenz, Switzerland) include band space of 10 mm, chamber saturation time of 30 min, chromatogram run of 8 cm and optimized at room temperature of 25±2°C and relative humidity of 60±5%. The mobile phase was methylene chloride: methanol (1:1, v/v) with densitometric analysis at 330 nm in the absorbance mode with Camag TLC scanner III operated by winCATS software (Version 1.2.0).

Standard solutions and spiked samples

Stock solution of moxi (1.2 mg mL^{-1}) was prepared by dissolving 24 mg in 20 mL of methanol. Working solutions ranging from 0.2-1.2 mg mL^{-1} were prepared by properly diluting stock solution with methanol. One microliter of

each working solution of moxi was used to spike aqueous and vitreous humor samples (10 µL) in order to obtain calibration standards ranging from 20-120 ng μL^{-1}. QC samples were prepared at concentration of 20 (low QC), 60 (medium QC) and 120 (high QC) ng μL^{-1}. Each level was divided into aliquots of 10.0 µL and immediately frozen at –20°C.

Ocular fluid sample preparation

Prior to analysis, aqueous and vitreous humor was thawed at room temperature for about 10 minutes. Ten µL of aqueous and vitreous humor calibration standards and QC samples were transferred into vials and mixed with 1.0 mL of extracting solvent (mixture of methanol: aqueous solution of EDTA, 9:1). After vortex mixing for 1 minute, the samples were centrifuged (5 min, > 2000 g). The supernatant was transferred to new tubes and the solvent was evaporated at 37°C under nitrogen stream. The samples were reconstituted with 10 µL of methanol. One microliter of each sample was spotted on the TLC plate to obtain calibration range of 20-120 ng $spot^{-1}$.

Preparation of Moxi-MNPs

The nanoparticles were prepared by a modified coacervation method as reported by Calvo et al. [24]. Briefly, the solution of PLGA (4.0%, w/v) in acetone was sprayed into the CS solution (0.2%, w/v) containing moxi (0.4%, w/v) and polyvinyl alcohol (PVA), under continuous magnetic stirring at 2000 rpm for 1h. PVA (1.0% w/v) acted as a stabilizer and aid dispersion of nanoparticles. Nanoparticles were formed as a result of the interaction between negative charged carboxylic groups of PLGA and positively charged amino groups of CS. Nanoparticles were collected by centrifugation (REMI Corporation, India) at 18,000 rpm for 30 min at 4°C. The droplet size and zeta potential were measured using a Zetasizer Nano-ZS90 (Malvern, U.K.).

Validation Procedures

1. Selectivity

The selectivity of the method was investigated by assessment of potential interferences from endogenous matrix constituents using the HPTLC conditions described above for monitoring moxi. All the samples were cleaned up as described in sample preparation section and the R_f values of endogenous compounds in the matrix were compared with those of moxi.

2. Detection and quantitation limits

In order to estimate the detection limit (DL) and quantitation limit (QL), blank methanol was spotted six times following the same method as explained in section 2.2 and the standard deviation (σ) of the magnitude of analytical response was determined. The DL was expressed as 3.3σ/slope of moxi calibration curve, whereas QL was expressed as 10σ/slope of moxi calibration curve.

3. Accuracy and precision

The intra-day precision and accuracy of the assays was evaluated by performing replicate analyses (n=6) of QC samples (20, 60 and 120 ng $spot^{-1}$) in ocular fluids. The inter-day precision and accuracy of the assay was determined by repeating the intra-day assay on three different days. Precision was expressed as the percentage coefficient

variation (CV, %) of measured concentrations for each calibration level whereas accuracy was expressed as percent recovery [(moxi found / moxi applied) x 100].

4. Extraction efficiency

Extraction efficiency was evaluated by analyte determination in six different aqueous and vitreous humor samples at 20, 60 and 120 ng spot^{-1}. They were analyzed in:

• Set 1: standard mixtures: aqueous and vitreous humor samples from six rabbits (10 µL) were vortexed mixed with 1.0 mL of extracting solvent. Samples were centrifuged (5 min, > 2000 g) and the upper organic phase was obtained. Then moxi was spiked to achieve the concentration of 2.0, 6.0 and 12.0 µg mL^{-1}. Ten microliter of each sample was spotted on the TLC plate to obtain concentration range of 20, 60 and 120 ng spot^{-1}.

• Set 2: aqueous humor mixtures: aqueous and vitreous humor samples (10 µL) from six rabbits were spiked with moxi and vortexed mixed with 1.0 mL of extracting solvent. Samples were centrifuged (5 min, > 2000 g) and the supernatant was transferred to new tubes and the solvent was evaporated at 37°C under nitrogen stream. The aqueous and vitreous humor samples were reconstituted with 100 µL of methanol. Ten microliter of each sample was spotted on the TLC plate to obtain concentration range of 20, 60 and 120 ng spot^{-1}.

5. Robustness and Ruggedness

Robustness was studied in triplicate at a concentration level of 60 ng spot^{-1}. The effect on the result was examined by introducing small changes in mobile phase composition, mobile phase volume, duration of chamber saturation, percentage of EDTA solution, pH of EDTA solution and time from spotting to chromatography. In order to assess the ruggedness of the method, a solution of 60 ng spot^{-1} was prepared and analyzed on day 0 and after 6, 12, 24, 48 and 72 h. Data was treated to calculate % R.S.D to assess robustness and ruggedness of the method.

6. Stability Studies

For stability of moxi in ocular fluids, aqueous and vitreous humor samples were spiked with moxi and stored at 20°C, 4°C and −20°C. Stability was assessed for upto 12 h at −20°C, for upto 2 days at 4°C and for upto two week at −20°C. Freeze-thaw stability was determined by assessing samples after three freeze-thaw cycles. At each cycle, samples were stored at −20°C for 24 h and then left to thaw completely at room temperature for 4 h. This cycle was repeated three times. Stock solution stability of the drug in methanol was assessed at 4°C and 20°C.

Pharmacokinetic studies

A freshly prepared and lyophilized Moxi-MNP formulation (equivalent to 0.5% moxi) was dispersed in pH 7.2 buffered isotonic vehicle. Moxi solution containing an equivalent amount of moxi was prepared in the same vehicle. Pharmacokinetics studies were performed New Zealand Albino rabbits (2.0-2.5 kg). The animal protocol was reviewed and approved by Institutional Animal Ethics Committee, Jamia Hamdard (approval no: 469) and their guidelines were followed. Two groups of seven animals received single instillation of moxi solution or Moxi-MNP formulation. One animal (two eyes) was used for each time point for each formulation tested; instillation was, therefore, received in both eyes of each rabbit. Approximately 100µl of aqueous and vitreous humor was collected in eppendorf tubes before drug administration and post-treatment at stipulated time points. All aqueous and vitreous

samples were sealed and stored at −20°C until analysis. The samples were extracted as mentioned in the section 2.4 and 10 µL of each sample was spotted on TLC plate. The time course of moxi in the ocular fluid was characterized by measurement of several different parameters. The time at which the drug first detected in ocular fluids and time after which it could no longer be detected was recorded. The maximum drug concentration and the time for maximum concentration were also recorded. Student t-test was used and $P<0.05$ used as an indication of statistical significance.

RESULTS

Characterization of Moxi-MNPs

The prepared Moxi-MNPs appeared spherical and uniform (polydispersity value, 0.115) with mean droplet size of approximately 202.5 nm diameter as measured using photon correlation spectroscopy. The positive zeta potential value of the particle (+32.5mV) indicates enhance interaction with negatively charged cornea.

Method validation

1. Linearity and sensitivity

Under the described chromatographic conditions, and in both ocular fluids, moxi was well resolved at R_f 0.35. The linear regression data for the calibration curves (n = 6) in aqueous and vitreous humor showed a good linear relationship over concentration range 20–120 ng spot^{-1} with the correlation coefficient (r^2) better than 0.99. The peak area vs. concentration fitted well to a straight line, with the following equations for the calibration curves: y = (2920.32±16.81)x + (3853.38±14.92) and y = (2750.12±19.80)x + (3618.73±12.32) in aqueous and vitreous humor, respectively. No significant difference was observed in the slopes of standard curves (ANOVA, $P > 0.05$). DL and QL were found to be 1.1 and 3.5 ng spot^{-1}, respectively, indicating adequate sensitivity of the method.

2. Selectivity and specificity

Method specificity towards endogenous blank aqueous and vitreous humor from each of the six rabbits was tested. These samples were pre-treated according to the sample preparation procedure, apart from addition of the drug. Chromatograms obtained from blank (drug-free) aqueous humor (Fig.1a) and vitreous humor (Fig.2a) were compared with aqueous humor spiked with moxi (Fig.1b) and vitreous humor spiked with moxi (Fig.2b) to show the specificity and selectivity of the proposed procedure. No significant peak was observed at or near the R_f of moxi, suggesting that the extraction procedure was capable of obtaining highly purified samples which in turn ensured a high selectivity of the HPTLC method.

3. Precision and accuracy

Table 1 summarizes the precision and accuracy of the moxi assay determined at concentration of 20, 60 and 120 ng spot^{-1}. For both the ocular fluid studied, the intra day precision was equal to or less than 5.3% and inter day precision was equal to or less than 5.7%. The intra- and inter-day accuracy ranged from 97.7–104.0% for the matrices studied.

4. Extraction efficiency

The extraction efficiency (EE) was evaluated by comparing the results of analysis of both sets of samples as follows: EE (%) = 100* (2)/ (1); where (1) is the mean peak area for concentration of set 1, and (2) is the mean peak area for

concentration of set 2. The overall extraction efficiency was 98.2 ± 2.6% in aqueous humor and 97.6 ± 3.4% in vitreous humor. High recoveries with low variability were found to be consistent over the calibration range and consequently the published method was proved to be reliable.

5. Stability

Moxi was found to be stable in ocular fluids for 12 h at 20°C, for 2 days at 4°C and for two week at −20°C with average recovery of 95.7, 96.2 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 methanol revealed no significant losses for atleast 5 days at 20°C and for 2 weeks at 4°C.

Pharmacokinetic study

Table 2 lists the concentration of moxi in aqueous and vitreous humor after single instillation of Moxi-MNPs and moxi solution into the rabbit eye. With both formulations moxi was detectable in aqueous humor 0.5 h after instillation. The Moxi-MNPs ensures effective levels of drug in the rabbit aqueous humor for atleast 24 h (214 ± 19.09 ng mL^{-1}). In the group treated with the solution the aqueous levels of moxi were undetectable after 2 h. The mucoadhesive Moxi-MNPs formulation appears to offer significant sustaining of drug release in the aqueous humor compared with control solution. The moxi levels obtained from the Moxi-MNPs formulation were significantly higher at 2, 4, 8, 12 and 24 h (P<0.01). Moxi-MNPs formulation also improved the drug penetration into the vitreous humor. With the solution formulation no moxi was detectable in vitreous humor whereas for Moxi-MNPs formulation moxi was detectable in vitreous humor at 2 h (140 ± 4.14 ng mL^{-1}) and 4 h (110 ± 12.5 ng mL^{-1}) time point. This is in accordance with previously reported results where vitreous concentrations were achieved through nanoparticle formulation [25]. In our preliminary analysis of human ocular fluids, the levels of the moxi in aqueous humor were significantly higher than in vitreous humor (P<0.05). These in vivo results are particularly interesting because they show that the Moxi-MNPs formulation increases drug bioavailability to the eye compared to drug solution. Moreover, good results are obtained in prolonging drug residence time in the eye by administration of Moxi-MNPs, probably because of their mucoadhesive properties.

DISCUSSION

The HPTLC procedure was optimized with a view to quantify the moxi in ocular fluids. Initially, the mobile phase of HPTLC system reported for degradation studies of moxi [21] was attempted but peak tailing and low recoveries were observed. Moxi, a fourth generation fluoroquinolone, with carbonyl and carboxyl groups tends to form complex with metal ions present in ocular fluids (Cu, Fe, Zn) and on silica plates (Ca, Al), consequently resulting in low recoveries and peak tailing. Different chelating agents such as EDTA, citric acid, oxalic acid and sodium diethanol glycine were tried as a component of mobile phase and extraction solvent. The use of TLC plates sprayed with 5% (w/v) solution of disodium EDTA (pH 9) allows us to prevent the formation of moxi-metal complexes and improves peak symmetry. Ideally, aqueous humor contains much smaller amount of proteins compared to plasma and therefore simple extraction with methanol enabled no interfering substances and impurities. However, for moxi extraction from ocular fluids, direct protein precipitation with methanol gave low recovery. Addition of aqueous solution of EDTA to methanol increases the recovery of moxi. Finally, a combination of methanol and aqueous solution of EDTA (9:1) effectively eliminates the interfering material and gave the best recovery for moxi. Under the chromatographic

conditions described in our study, a sharp and well-defined symmetrical peak at R_f 0.35±0.02 for moxi was observed. The R_f 0.35 obtained by our method is significantly different from that obtained by previously reported stability indicating HPTLC method (R_f 0.52) of moxi [21]. The reduction in R_f value is due to different mobile phase, methanol: dichloromethane (1:1, v/v), used in our assay and might be due to decreased interaction of moxi with EDTA sprayed TLC plates. Densitometric analysis at 330 nm improved the detection sensitivity, specificity and minimizes interferences from ocular fluids that may occur at lower wavelengths. The QL (3.5) and DL (1.1) for moxi in this assay represents a nearly 10-fold increase in sensitivity over a previously reported HPLC assay for the measurement of moxi levels in ocular fluids [18]. The QL is certainly sufficient for ocular fluid pharmacokinetics and may also be suitable for plasma pharmacokinetics.

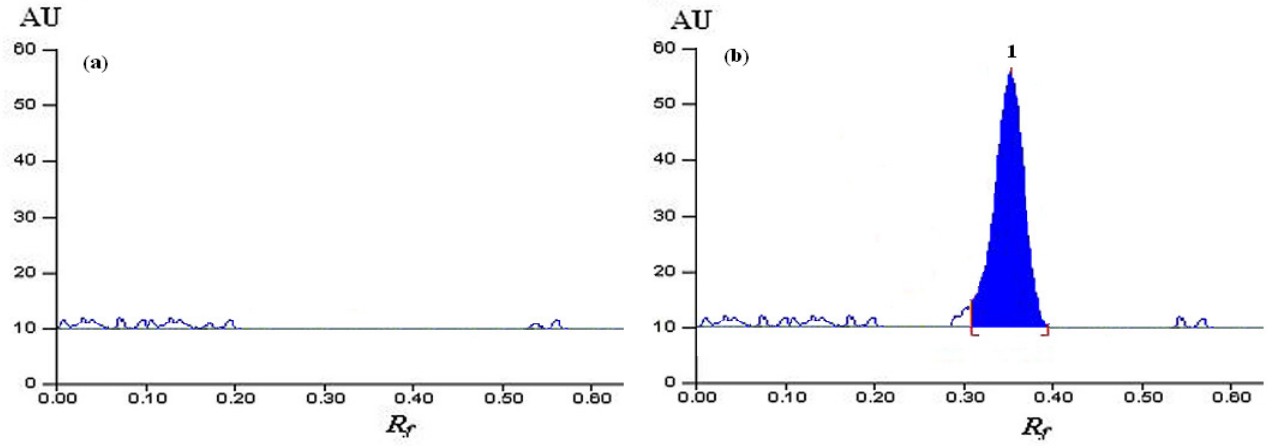

Fig. 1 Chromatograms of (a) drug-free aqueous humor, (b) aqueous humor spiked at a concentration of 60 ng spot^{-1}. Peak 1: moxi, *R*f 0.35; detector wavelength, 330 nm.

Also, the total analytical run-time using the current method was considerably shorter when compared with the reported run-time of 16.7 min for each ocular fluid samples in an earlier study [18]. Even with HPLC method with shortest chromatographic time (< 10 min) would be able to process a maximum of 6 samples per hour. In contrast, this HPTLC method should be able to assay large batches of samples in parallel. Further, the current HPTLC assay requires minimal sample preparation and circumvents the extensive derivatization and processing of samples commonly required. No interfering peaks were observed in the blank aqueous humor chromatogram, indicating the efficient clean up method used. The present method, devoid of internal standard, is robust and % CV of the moxi in ocular matrices was <5.3%. In addition, the method offers significant advantages in terms of greater sensitivity, rapid analysis and minimal sample preparation requirements. Stability studies with moxi show that the samples could be stored frozen (- 20°C) for at least 14 days and could withstand three freeze/thaw cycles without compromising their integrity. The method was successfully applied to study and compare ocular fluid levels obtained after instillation of single dose of moxi solution and Moxi-MNPs formulation to rabbit eye. The Moxi-MNPs formulation appears to offer sustained drug release to aqueous humor and improved drug penetration to vitreous humor. The in vivo results show that the Moxi-MNPs increases ocular drug bioavailability and prolonged drug residence time in the eye.

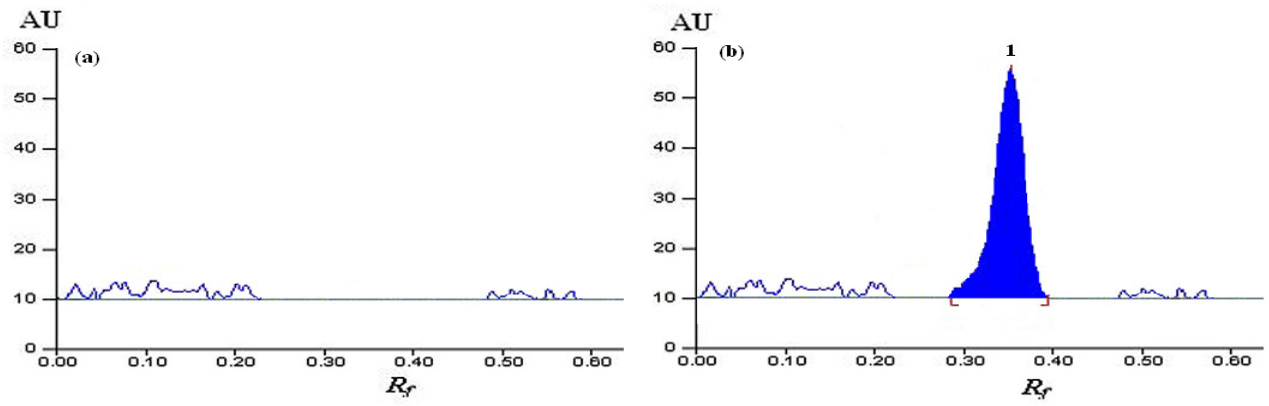

Fig. 2 Chromatograms of (a) drug-free vitreous humor, (b) vitreous humor spiked at a concentration of 60 ng spot^{-1}. Peak 1: moxi, *Rf* 0.35; detector wavelength, 330 nm.

CONCLUSION

In conclusion, HPTLC assay of moxi would appear to be a very useful addition to the currently used techniques for measuring moxi in ocular fluids since it allow rapid assay with the use of less costly resources and merits further evaluation in studies examining drug pharmacokinetics.

Table 1 HPTLC Validation data: precision and accuracy

	Intra-day reproducibility			Inter-day reproducibility		
Moxi applied (ng spot^{-1})	Moxi found (ng spot^{-1}) (mean±sd)	Precision [a] (CV, %)	Accuracy [b] (%)	Moxi found (ng spot^{-1}) (mean±sd)	Precision [a] (CV, %)	Accuracy [b] (%)
Aqueous humor						
20	19.7 ± 0.9	4.6	98.5	19.2 ± 1.1	5.7	96.0
60	60.8 ± 2.6	4.3	101.3	58.7 ± 2.9	4.9	97.8
120	118.1 ± 2.9	2.4	98.4	117.9 ± 4.2	3.6	98.2
Vitreous humor						
20	20.8 ± 1.1	5.3	104.0	19.8 ± 0.8	4.0	99.0
60	59.1 ± 2.4	4.1	98.5	58.6 ± 3.0	5.1	97.7
120	122.1 ± 3.7	3.0	101.8	120.3 ± 3.5	2.9	100.3

[a] Precision as coefficient of variation (CV, %) = (standard deviation/ moxi found) x 100

[b] Accuracy = (moxi applied/ moxi found) x 100

Table 2 Concentration of moxi in ocular fluids after single instillation of moxi solution and Moxi-MNPs formulation into rabbit eye

Rabbit Number	Time (h)	Moxi concentration[a] (ng mL^{-1})			
		Moxi solution		Moxi-MNPs formulation	
		Aqueous humor	Vitreous humor	Aqueous humor	Vitreous humor
1	0.5	120 ± 14.14	n.d.	146 ± 5.66	n.d.
2	1.0	417 ± 15.55	n.d.	377 ± 12.73	n.d.
3	2.0	215 ± 25.35	n.d.	378* ± 11.31	140* ± 4.14
4	4.0	n.d.	n.d.	401.5* ± 2.12	110* ± 12.5
5	8.0	n.d.	n.d.	440.5* ± 9.26	n.d.
6	12.0	n.d.	n.d.	338* ± 22.63	n.d.
7	24.0	n.d.	n.d.	214* ± 19.09	n.d.

[a] Values are mean ± s.d. of results from two eyes;

* $P < 0.01$ significant difference compared with moxi solution; n.d., not detectable

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