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A LC-MS/MS validated method for determination of azithromycin in human tears and its application to an ocular pharmacokinetic study

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A rapid and sensitive method for the quantitative analysis of azithromycin in human tears by LC-MS/MS was developed and validated. Following extraction from collected Schirmer tear strips by methanol-water (4:1, v/v), the analyte and IS (azithromycin-d3) were separated on a Waters Atlantis™ dC_{18} column (2.1 mm × 30 mm, 3 μm) by gradient elution with 0.1% (v/v) formic acid in methanol-water (1:9) and methanol-acetonitrile (9:1) as the mobile phase. Electrospray ionization in positive ion mode and MRM were used to monitor the ion transitions at *m/z* 749.6→591.6 (azithromycin) and 752.4→594.4 (azithromycin-d3). The results indicated that the method had excellent sensitivity and specificity. The analyte appeared to have good linearity in the range of 5-1000 ng/mL. Both the intra-batch and inter-batch precisions (in terms of RSD) were <10%, and the accuracies (in terms of RE) were within ±15%. The lower limit of quantification, matrix effect, extraction recovery, stability and dilution integrity were also evaluated and satisfied the validation criteria. Artificial tears served as the surrogate matrix, and no matrix difference was found when compared with that of real human tears. Finally, this method was successfully applied in an ocular pharmacokinetic study in healthy volunteers following instillation of azithromycin eyedrops.

1. Introduction

Recent years have seen the development of eyedrops containing 1% azithromycin in a bioadhesive ocular drug-delivery system that stabilizes and sustains the release of azithromycin to the ocular surface. This leads to a longer drug residence time, less frequent dosing, and increased patient compliance compared to those of conventional treatment methods (Opitz and Harthan 2012). Some clinical studies have evaluated the efficacy and safety of 1% azithromycin eyedrops in the management of ocular conditions, such as bacterial conjunctivitis and blepharitis, in both paediatric and adult populations (Abelson et al. 2007; Protzko et al. 2007; Torkildsen et al. 2011).

Azithromycin eyedrops offer a simplified dosing regimen: the therapy involves administering seven drops over a period of five days, as the high bactericidal activity of the drops is sustained overnight in tears. Although effective ocular maintenance has been documented, the disposition of azithromycin in the eye has not been fully explored ((Bowman et al. 2009; Chiambaretta et al. 2008; Wu et al. 2019). To construct robust ocular concentration-time profiles, an accurate and reliable quantitative analysis method of azithromycin in human tears is needed.

To the best of our knowledge, most published papers on determination methods of azithromycin were developed using human plasma samples (Ben-Eltriki et al. 2013; Choemunng and Na-Bangchang 2010; Filist et al. 2014). A LC-MS method for azithromycin determination in human tear samples was described, but the method is rather complex with a lower limit of quantitation (LLOQ) of only 0.17 μg/g (Chiambaretta et al. 2008). This paper describes the development and validation of a new LC-MS/MS method for the determination of azithromycin in human tears. The analytes were extracted from Schirmer tear strips. Ocular matrices, including tears, are considered to be rare matrices and are often difficult to source. Commercially available artificial tear solution is frequently used as a blank matrix (Hirosawa et al. 2015; Maksić et al. 2017; Shiokawa et al. 2019). To demonstrate its suitability for use, one set of quality controls (QCs) prepared in an authentic matrix can be quantified with a surrogate calibration standard curve during validation (Heinig et al. 2017). Therefore, a bridge test between artificial tear solution and collected real human tears was included in the method validation of this study. The assay was further applied to detect the azithromycin concentration in tear samples from an ocular pharmacokinetic study in healthy volunteers.

2. Investigations and results

2.1. Method validation

2.1.1. Mass spectrometric method conditions

The MS method was screened and optimized by a systematic approach. Under the assistance of a syringe pump that injected standard solutions into the mass spectrometer, the precursor and product ions were determined. The highest ion abundance was achieved by optimizing the source/gas and compound parameters. Table 1 shows the optimal ionization conditions. Product spectra, chemical structures and proposed fragmentation patterns of azithromycin and azithromycin-d3 are shown in Fig. 1.

Table 1: Optimized mass-to-charge (m/z) and MS parameters

Analytes	*m/z*	DP (V)	EP (V)	CE (V)	CXP (V)
Azithromycin (quantitation)	749.6→591.6	80	10	40	15
Azithromycin (qualification)	749.6→158.1	80	10	55	13
Azithromycin-d3 (IS)	752.4→594.4	110	10	41	17

DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential.

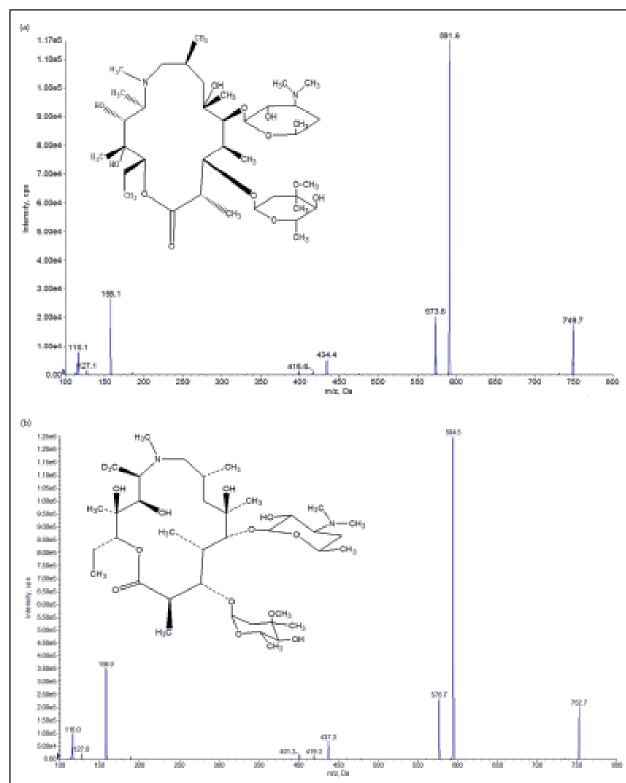

Fig. 1: Mass spectra and chemical structures of azithromycin (a) and the internal standard, azithromycin-d3 (b).

2.1.2. Selectivity, linearity and sensitivity

For the system suitability experiment, the precision (CV) was 0.27% for the retention time and 2.88% for the area ratio of azithromycin and azithromycin-d3. The LC-MS/MS method was found to be selective and sensitive for azithromycin, and the LLOQ was 5 ng/mL for azithromycin, with signal-to-noise ratios higher than

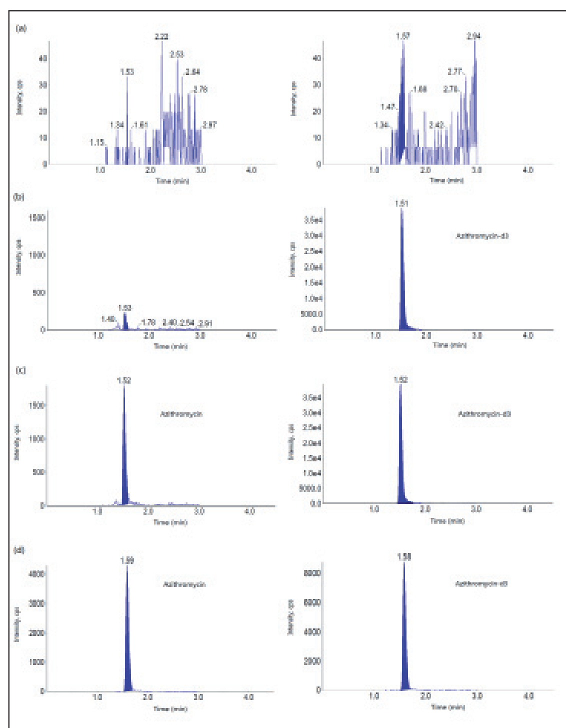

Fig. 2: Multiple reaction monitoring ion chromatograms of (a) a double blank tear sample (without analyte and IS), (b) a blank tear sample with azithromycin-d3 (IS), (c) azithomycin at LLOQ (5 ng/mL) and (d) an authentic tear sample after instillation of single-dose azithromycin eyedrops.

20. The selectivity of the method was ascertained from the MRM (multiple reaction monitoring) ion chromatograms shown in Fig. 2. In each validation batch, seven calibration standards were analysed, and the calibration curve showed good linearity in the range of 5–1000 ng/mL for azithromycin. The regression coefficients of all of the calibration curves were >0.99. Back-calculated concentrations of calibration standards for azithromycin are listed in Table 2.

Table 2: Back-calculated concentrations of calibration standards for azithromycin (linear weighted 1/x^2, n=8)

Item	S1	S2	S3	S4	S5	S6	S7	*r*
Nominal concentration (ng/mL)	5	10	50	100	400	800	1000	
Mean	5.05	9.77	50.2	105	391	807	978	0.998
RSD (%)	3.1	5.5	6.8	2.4	3.7	3.4	4.4	0.1
RE (%)	0.9	-2.3	0.3	4.6	-2.2	0.9	-2.2	

2.1.3. LLOQ, precision and accuracy

The intra- and inter-batch precision and accuracy data for the determination of azithromycin at the LLOQ and three QC levels are summarized in Table 3. Both the intra-batch and inter-batch precision (RSD%) of the QC samples were ≤ 10%, while the accuracy (RE%) ranged from -15% to +15%. The results demonstrated that the precision and accuracy satisfied acceptable limits. This method was reliable and reproducible for the determination of azithromycin in human tears.

Table 3: Intra- and inter-batch accuracy and precision of quality control samples of azithromycin

Batch	Item	LLOQ	LQC	MQC	HQC	Dilution QC
Nominal concentration (ng/mL)		5	15	75	750	3000
Batch 1 (n=6)	Mean	4.66	14.1	67.0	671	2752
	RSD (%)	5.3	4.2	2.5	3.3	3.7
	RE (%)	-6.7	-5.8	-10.7	-10.6	-8.3
Batch 2 (n=6)	Mean	4.92	15.2	69.5	712	
	RSD (%)	5.1	1.8	3.7	3.6	
	RE (%)	-1.6	1.5	-7.3	-5.0	
Batch 3 (n=6)	Mean	4.31	15.0	70.6	695	
	RSD (%)	4.1	3.8	6.0	4.0	
	RE (%)	-13.8	0.0	-5.8	-7.3	
Batch 4 (n=6)	Mean	4.91	15.4	73.0	755	
	RSD (%)	5.8	4.5	2.3	2.1	
	RE (%)	-1.7	3.0	-2.6	0.7	
Batch 5 (n=6)	Mean		14.7	71.9	711	
	RSD (%)		3.9	2.6	4.2	
	RE (%)		-2.2	-4.2	-5.3	
Batch 6 (n=6)	Mean		15.4	81.9	732	
	RSD (%)		8.5	1.8	2.5	
	RE (%)		2.6	9.2	-2.4	
Inter-batch	Mean	4.70	15.0	72.3	713	
	RSD (%)	7.2	5.5	7.3	4.9	
	RE (%)	-6.0	-0.1	-3.6	-5.0	

2.1.4. Matrix effect and extraction recovery

The matrix effect of azithromycin at the LQC, MQC and HQC concentrations was between 100.40 and 103.93% and that of the IS (internal standard) was 99.59±2.42%. The overall mean extraction recovery of azithromycin at three different QC concentrations was 97.53±1.90% and that of the IS was 95.74±3.44%. The detailed

results of the matrix effect and extraction recovery are shown in Table 4. In addition, the mean values of carryover in the double blank samples were 15.90% for the analyte and 0.11% for the IS compared with the LLOQ sample in the same batch.

Table 4: Matrix effect and extraction recovery of azithromycin in tears (n=6)

Spiked concentration (ng/mL)	Matrix effect		Extraction recovery	
	Mean±SD (%)	RSD (%)	Mean±SD (%)	RSD (%)
15	103.9±1.7	1.6	96.6±3.7	3.8
75	100.4±2.5	2.5	99.7±3.1	3.1
750	100.4±2.2	2.2	96.2±2.1	2.2
200 (IS)	99.6±2.4	2.4	95.7±3.4	3.6

2.1.5. Stability

The short-term, autosampler, freeze-thaw and long-term stability results are shown in Table 5. Azithromycin had good stability in tear samples (Schirmer strips) at room temperature for at least 5 h and at 2-8 °C for up to 15 h. They were also stable in frozen tear samples (-40 °C) for up to 176 days. Azithromycin tear samples remained stable following three freeze-thaw cycles from -40°C to room temperature. Moreover, the processed samples remained stable after being placed in the autosampler (4 °C) for at least 46 h.

Table 5: Stability assessments for azithromycin in tears and processed samples (n= 6)

Stability types	Nominal concentration (ng/mL)	Mean	RSD (%)	RE (%)
Short-term (room temperature for 5 h)	15	15.7	13.8	4.5
	750	752	1.9	0.3
Short-term (2-8°C for 15 h)	15	14.3	3.4	-4.6
	750	669	3.4	-10.8
Autosampler (4°C for 46 h)	15	14.3	4.0	-4.9
	75	65.2	2.5	-13.0
	750	641	2.5	-14.6
Freeze-thaw (three cycles)	15	15.2	4.7	1.5
	750	718	2.0	-4.3
Long-term (-40°C for 15 days)	15	14.4	5.5	-4.0
	750	713	5.8	-4.9
Long-term (-40°C for 176 days)	15	16.7	5.7	11.0
	750	731	3.3	-2.5

2.1.6. Dilution integrity

Following a 40-fold dilution, the mean precision (RSD) and accuracy (RE) at three different QC concentrations were <10% (Table 3). The results indicated that samples could be diluted 40-fold when their concentrations were higher than the upper limit of quantification (ULOQ) level. This expanded the quantification range to 5–3000 ng/mL for azithromycin determination.

Table 6: Surrogate matrix bridge test for azithromycin in real human tear samples

	Item	LLOQ	LQC	MQC	HQC
Nominal concentration (ng/mL)		5	15	75	750
Bridge test (n=6)	Mean	4.31	15.0	70.6	695
	RSD (%)	4.1	3.8	6.0	4.0
	RE (%)	-13.8	0.0	-5.8	-7.3

2.1.7. Surrogate matrix bridge test

Six duplicate samples at the LLOQ and three QC levels were prepared in real human tears and were quantified with the calibration standard curve constructed with the artificial tear solution. Both the mean precision (RSD) and accuracy (RE) were <10% (Table 6). No matrix difference was found, and the artificial tear solution could be used as an appropriate matrix in the determination of azithromycin in human tears.

2.2. Method application

The validated method in this paper was used to determine azithromycin in human tear samples following single-dose topical administration of azithromycin eyedrops (2.5 mL/25 mg) in healthy Chinese volunteers. Forty-two subjects had one azithromycin eyedrop instilled into each eye, leading to a total of 84 tear samples that were available to test the applicability of this method. The mean tear concentration vs. time profile of azithromycin in healthy volunteers is presented in Fig. 3.

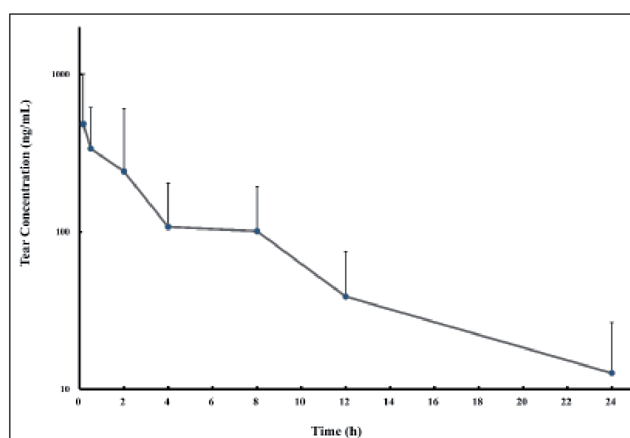

Fig. 3: The mean tear concentration-time profile of azithromycin following single-dose topical administration of azithromycin eyedrops (2.5 mL/25 mg) in 42 healthy volunteers.

2.3. Conclusion

The established method is rapid, sensitive, accurate and reliable with good specificity and stability for the determination of azithromycin concentrations in human tears. The LC-MS/MS conditions were optimized to improve the sensitivity and robustness of the method. The results were successfully validated with the quantitative analysis of azithromycin in samples from healthy volunteers after instillation of azithromycin eyedrops. This is the first published assay validated for the determination of azithromycin in human tears and met the requirements as described in related international guidelines and facilitated the study of ocular pharmacokinetic characteristics in human subjects.

3. Discussion

In this study, the analyte was extracted directly from Schirmer tear strips. Various extraction agents and different proportions were considered. An optimal mass spectrometric response and only mild ion suppression were observed in methanol-water at 4:1 (v/v).

Compared with those after implementation of other MS-compatible additives, a preferable peak shape and mass response were provided by adding 0.1% formic acid into both the aqueous and organic portions of the mobile phase. The analyte and IS were determined simultaneously with a run time of 4.5 min.

The standard curves constructed for azithromycin in the samples after extraction ranged from 5 to 1000 ng/mL. Since the tear concentration of azithromycin varies greatly, a wide calibration range was needed. We set the LLOQ at 5 ng/mL to ensure that the tear concentration could be detected after 24 h. The ULOQ was set to 1000 ng/mL to facilitate linear fitting. Then, dilution integrity was validated to expand the quantification range to 3000 ng/mL.

An isotopically labelled IS (azithromycin-d3) was used in this study, which had a similar structure to that of the analyte but did not interfere with it. Both the analyte and IS had strong positive ion signals and experienced no matrix effect. The isotopically labelled IS was preferable for the calibration of azithromycin in tears compared to other types of ISs, such as erythromycin (Wu et al. 2019), imipramine (Ben-Eltriki et al. 2013), roxithromycin (Choemunng and Na-Bangchang 2010), etc.

For the ocular pharmacokinetic study, this assay was adequately sensitive for monitoring the tear concentration of azithromycin up to 24 h after single-dose topical instillation. The observed mean $C_{24\ h}$ value was 12.6 ng/mL, and the lowest concentration was 0.576 ng/mL, which were both comparable with those reported in previous studies (Chiambaretta et al. 2008; Wu et al. 2019).

4. Experimental

4.1. Chemicals and reagents

An azithromycin standard (purity 94.4%) was purchased from the China Research Institute of Food and Drug Verification (Beijing, China); azithromycin-d3 (IS, purity 98.0%) was purchased from TRC Pharmaceutical Standards (Toronto, Canada).

Acetonitrile (ACN, HPLC-grade) and methanol (MeOH, HPLC-grade) were purchased from Honeywell Co., Ltd (Muskegon, MI, USA). Formic acid (HPLC-grade) was purchased from Dikma Pure (Richmond, Hill, USA). Dimethyl sulfoxide (DMSO) was purchased from Sinopharm Chemical Reagent Co. Ltd (Beijing, China). Ultrapure water (≥18.2 MΩ·cm) was produced by a Milli-Q Reagent water purification system (Millipore, Bedford, MA, USA).

An artificial tear solution, Tears Naturale®, produced by Alcon Laboratories, Inc. (Fort Worth, TX, USA), was used as the surrogate blank matrix for real human tears in the present study. Sterile Schirmer tear strips were produced by Jingming New Technological Development Co., Ltd (Tianjin, China).

4.2. Instrumentation and conditions

Liquid chromatography was performed on an Agilent 1200 HPLC system (Agilent Corp., Milford, MA, USA), and mass spectrometric detection was carried out on an API 4000 triple quadrupole-linear ion trap mass spectrometer (AB SCIEX, Framingham, MA, USA), which was fitted with a TurboIon Spray ionization source. Analytical separation was performed on a Waters Atlantis™ dC18 column (2.1 mm × 30 mm, 3 μm) at a temperature of 20 °C controlled by a column heater.

Processed samples were analysed using reversed-phase liquid chromatography. Chromatographic separation was performed by gradient elution using a solution with methanol-water-formic acid (10:90:0.1, v/v/v, solvent A) and methanol-acetonitrile-formic acid (90:10:0.1, v/v/v, solvent B). The mobile phase was maintained at 80% solvent A for 0.1 min after injection. The gradient changed linearly from 80 to 5% solvent A in 1.2 min and was then maintained at 5% solvent A for 1.5 min. Next, the proportion of solvent A was increased to 80% over 0.01 min and maintained for a 1.69 min re-equilibration of the column until the end of the chromatographic run. The flow rate was 0.45 mL/min, and the total run time for one sample was 4.5 min. The autosampler temperature was set to 4 °C with an injection volume of 10 μL.

Mass spectrometry was utilized for detection and was performed via an API-4000 mass spectrometer. The electrospray ionization (ESI) source was operated in positive ion mode with MS parameters as follows: source temperature, 450°C; nebulizer (nitrogen), 40 psi; turbo gas (nitrogen), 55 psi; curtain gas (nitrogen), 30 psi; collision gas, 4 psi; and ion spray needle voltage, 5000 V. Acquisition in MRM mode was performed.

4.3. Calibration standards and quality controls

Stock solutions of azithromycin and the IS were prepared in duplicate by dissolving the accurately weighed standards in a solution (DMSO-methanol, 1:1, v/v). The concentrations of azithromycin and the IS were 10 mg/mL and 1 mg/mL, respectively, for the preparation of calibration standards and QCs.

The IS working solution (200 ng/mL) was diluted from the stock solution with a solution of methanol-water (4:1, v/v). The intermediate working standard solutions were prepared at concentrations of 10, 20, 100, 200, 800, 1600 and 2000 μg/mL by dilution of the azithromycin stock solution with methanol-water (4:1, v/v). By diluting the intermediate working standard solutions with a blank artificial tear solution, the final concentrations of the azithromycin standard tear samples were 0.5, 1, 5, 10, 40, 80 and 100 μg/mL. Twenty microlitres of the standard tear samples were transferred and added to blank Schirmer strips and then extracted by 2000 μL methanol-water (4:1, v/v) containing the IS working solution (200 ng/mL arithromycin-d3) in 5 mL EP tubes. The final concentrations of the extracted standard calibration samples were 5, 10, 50, 100, 400, 800 and 1000 ng/mL.

QC spiked solutions were prepared at concentrations of 10, 30, 150 and 1500 μg/mL by diluting the primary azithromycin stock solution (10 mg/mL) from a separate reference material with methanol-water (1:1, v/v). The LLOQ and low, medium and high standard tear samples were prepared at concentrations of 0.5, 1.5, 7.5 and 75 μg/mL azithromycin, respectively, by diluting the QC spiked solutions with the blank artificial tear solution. After transferring 20 μL into Schirmer strips and extraction by 2000 μL methanol-water (4:1, v/v) containing the IS working solution, the final extracted QC samples were at concentrations of 5, 15, 75 and 750 ng/mL.

4.4. Sample preparation

The unknown tear samples were collected in 5 mL EP tubes and extracted by 2000 μL methanol-water (4:1, v/v) containing the IS working solution (200 ng/mL arithromycin-d3). After 10 min of ultrasonication and 10 min of thorough vortexing, the extracted unknown tear samples, calibration and QC samples were centrifuged at 4,000 rpm at 4°C for 5 min. Then, 10 μL of the supernatant was transferred to an autosampler vial and diluted with 190 μL of ultrapure water. Finally, 10 μL of the solution was injected into the LC-MS/MS system.

4.5. Method validation

Validation of this developed method was conducted according to the guidelines of the Food and Drug Administration (2013), European Medicines Agency (2011), China Food and Drug Administration and Chinese Pharmacopoeia Commission (2015), including linearity, LLOQ, precision, accuracy, matrix effect, extraction recovery, stability, dilution integrity and carryover. All data used for quantitation were collected and processed by Analyst software (version 1.6.2) of AB SCIEX (Concord, Ontario, Canada).

The linearity of this method was assessed over the respective calibration ranges of the analyte and IS. The calibration curves were constructed with seven concentrations. The linear regression of each calibration curve was performed with the nominal concentration being the explanatory variable (x) and the peak area ratio (y) of the analyte to IS being the dependent variable, of which the weighting factor was $1/x^2$. The bias of the calibration standards to the nominal concentrations in each calibration curve should be <15% except for at the LLOQ, for which the bias could be <20%. All calibration standards should meet the above criterion in each analytical run, and the correlation coefficient (r) should be >0.99.

LLOQ and three levels of QC samples, namely, LQC, MQC and HQC, were analysed to assess the precision and accuracy of the assay. For each QC concentration, analysis was performed in six replicates on each run. Precision and accuracy were denoted by RSD and RE, respectively. Both the inter- and intra-batch accuracy of LQC, MQC and HQC samples should be within ±15% of the corresponding nominal concentration, and the inter- and intra-batch precision should also be <15%. The accuracy and precision were required to be within ±20% for RE of the nominal concentration and <20% for RSD of LLOQ samples in the intra- and inter-batch assays.

The matrix effect was evaluated quantitatively by measurement of the matrix factor, a ratio of the analyte peak response in the presence of matrix ions to the analyte peak response in the absence of matrix ions, i.e., in solvent. The matrix effects for azithromycin were estimated by calculating the ratio of the peak area in the presence of matrix (extracted blank matrix spiked with the analyte) to the peak area in the absence of matrix (pure water used in place of tears). The RSD of the matrix effect should be ≤15%. The extraction recovery was assessed by comparing the average peak areas obtained from extracted QC samples with those of the equivalent post extracted spiked QC samples prepared using real human tears.

Freeze-thaw stability was evaluated after three freeze (-40 °C) and thaw (room temperature) cycles before sample preparation. Short-term stability was assessed by analysing QC samples kept at room temperature for 5 h and at 2-8 °C for 15 h. Autosampler stability was evaluated after the processed QC samples were placed in an autosampler (2-8 °C) for 46 h. Long-term stability was investigated by analysing the QC samples after storage at -40 °C for 15 days and 176 days.

To analyse the samples at a concentration above the ULOQ, extracted QC samples with azithromycin at 3000 ng/mL were diluted 40-fold with the artificial tear solution and then analysed. The precision and deviation from the nominal concentrations for six replicates after dilution should be ≤15%.

The double-blank tear samples that did not contain any analyte or IS were injected following the ULOQ samples to investigate the carryover of this method in carryover validation (six duplicates) and in each assay batch. For the analyte, the carryover should be <20% of the peak response of the corresponding LLOQ in the same batch. For the IS, the carryover should be <5% of the peak response of the corresponding LLOQ in the same batch.

The matrix difference between the artificial tear solution and real human tears was assessed through a bridge test. Six duplicates of QC samples at the relative concentrations of LLOQ, LQC, MQC and HQC were prepared using real human tear samples. These QC samples were quantitated against the calibration standard curve prepared with the artificial tear solution in the same run.

4.6. Application to an ocular pharmacokinetic study

This validated LC-MS/MS method was then applied to an ocular pharmacokinetic study in healthy volunteers following single-dose instillation of azithromycin eyedrops (2.5 mL/25 mg). Tears were sampled at seven time points: 0.17, 0.5, 2, 4, 8, 12, and 24 h after administration. Tear samples were collected using a Schirmer strip, which was hooked into the lower conjunctival sac over the inner one-third of the lower eyelid margin for 5 min or until tears covered the entire strip. Each Schirmer strip was weighed with an analytical balance before and after tear sampling. The tear samples were promptly stored at -40 °C until further analysis. The azithromycin concentration in 84 collected tear samples was then determined. Written informed consent was obtained from each subject prior to enrollment. Approval for the study was obtained from the ethics committee of Beijing Tongren Hospital.

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Conflicts of interest: none declared.

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