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Rapid, sensitive and selective liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the quantification of topically applied azithromycin in rabbit conjunctiva tissues

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ABSTRACT

A simple, rapid, sensitive and selective liquid chromatography–tandem mass spectrometry method was developed and validated for the quantification of azithromycin in rabbit conjunctiva tissues using roxithromycin as internal standard. Following a deproteinization procedure, the samples were eluted isocratically at a flow rate of 0.3 mL/min utilizing a mobile phase containing of 10 mM ammonium acetate (adjusted pH to 5.2 with 0.1% acetic acid)–methanol (18:82, v/v) and a SHISEIDO CAPCELL PAK C_{18} (3.0 mm × 75 mm, 3 μm). Azithromycin and its internal standard were measured by a triple-quadrupole mass spectrometer in the selected reaction monitoring (SRM) mode with precursor-to-product qualifier transition m/z 375 $[M+2H]^{2+} \rightarrow$ 591 and m/z 837 $[M+H]^{+} \rightarrow$ 679 respectively. The method demonstrated that good linearity ranged from 10 to 2000 ng/mL with $r = 0.9998$. The lower limit of quantification for azithromycin in conjunctiva tissues was 10 ng/mL with good accuracy and precision. The intra- and inter-day precision (RSD) values were below 15% and accuracy (%) ranged from 90% to 110% at all QC levels. The method was applicable to ocular pharmacokinetic studies of azithromycin.

1. Introduction

Azithromycin is a semi-synthetic macrolide antibiotic of the erythromycin group with a 15-membered azalactone ring (Fig. 1). Azithromycin appears to bind to the same receptor and has a very high tissue-to-blood concentration ratio with a half-life of 2–4 days in most tissues. And this may partly explain its outstanding antibiotic performance [1,2]. At present, azithromycin products in domestic market comprise almost all kinds of dosage forms and mainly used in respiratory infection, transmissible sex disease, cutaneous and soft tissue infection, etc. Disease incidence of conjunctivitis, especially those caused by Chlamydia trachomatis is very high in public nowadays. According to the data provided by WHO, there are about 84,000,000 people who had affected by trachoma. It is reported that the existent oral dosage form of azithromycin are suitable to treat the ocular infection such as conjunctivitis and other sensitive pathogens [3–5]. But when azithromycin was taken orally to cure ocular infection, it needs at least 1.0 g azithromycin per dose to ensure the drug content in aqueous humor, tear fluid and conjunctival coat reach the minimal inhibitory concentration (MIC). Thus not only will it waste much but it can bring on side-effects because of high drug level in every tissue [6,7]. Therefore, a new topical ophthalmic formulation of azithromycin with a low dosing frequency would be much more convenient for patients and thus ensure better compliance, thereby reducing the risk of selection of resistant bacteria. Since azithromycin has poor water solubility, azithromycin salt form is a pharmaceutically acceptable choice used to prepare its aqueous topical ophthalmic solution (EP 0677530, US Patent 6569443).

To increase the precorneal retention of drugs, mucoadhesive materials such as sodium hyaluronate, gellan gum, hydroxyl methylcellulose, polyvinyl alcohol, hydroxypropyl methylcellulose, carboxyl methylcellulose and polycarbophil, have been used to create a potentially long-acting effect in improving ocular drug bioavailability [8–12]. Currently, in order to heal bacterial conjunctivitis, USA InSite Vision company had developed an ophthalmic azithromycin dosage form—AzaSite by taking DuraSite as drug delivery system. This system forms a stable mucoadhesive matrix that stays in contact with the conjunctiva and delivers active drug to the ocular surface over a period of several hours [13,14]. In our lab, novel ophthalmic azithromycin dosage form was developed and investigated to cure ocular bacterial infections employing sodium hyaluronate which has bioadhesion property and mutual effect as mucoadhesive materials (CN 1814299A).

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In order to estimate the outstanding antibiotic performance in curing ocular infections, a sensitive analytic method for azithromycin to detect the concentration in conjunctiva tissues, which is formulated using mucoadhesive materials, should be established. The reported LC–MS [15] or LC–MS/MS analytical methods almost refer to the determination of azithromycin in plasma [16,17], which required either a complicated extraction and/or derivatization procedure [18–21], complicated bio-sample pretreatment or long analysis time [22,23], and may not well meet the requirement of desired throughput, speed and sensitivity in bio-sample analysis.

The subject of this paper is to develop a fast, selective and highly sensitive LC–MS/MS method with simple pretreatment procedures for the determination of azithromycin formulated using sodium hyaluronate as the mucoadhesive agent in rabbit conjunctiva tissues.

2. Experimental

2.1. Reagents and chemicals

Azithromycin reference standard (99.2% of purity) and roxithromycin (internal standard, I.S., 99% of purity) (Fig. 1) were obtained from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, PR China). Sodium hyaluronate (Eye-drop Grade, No. 20081104) was purchased from Freda Biochem Co. Ltd. (Shandong, China). Methanol of HPLC grade was obtained from Shandong Yuwang Sci-Tech Co. Ltd., China. And all other reagents were of analytical grade.

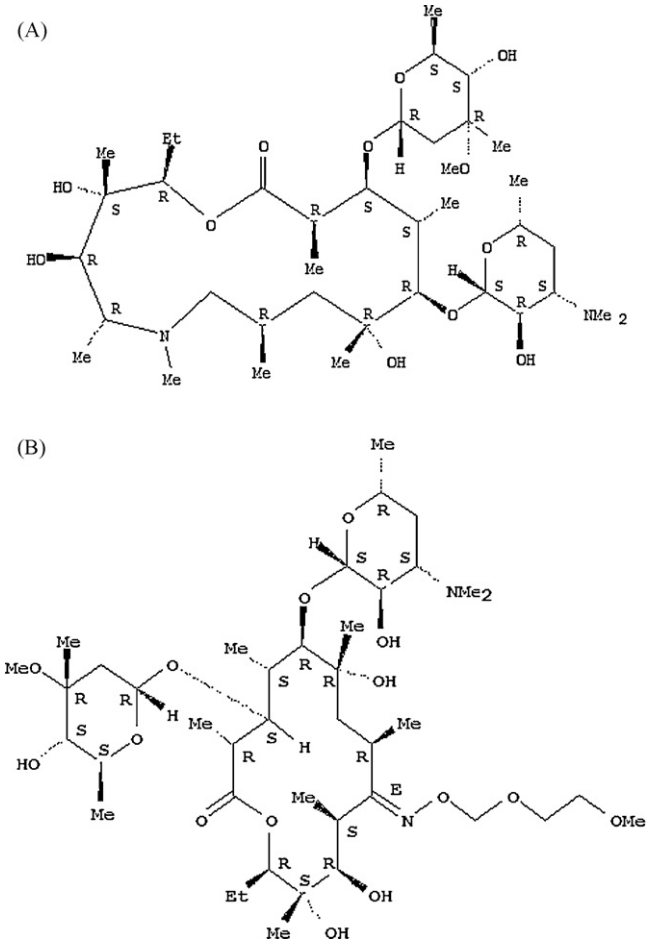

Fig. 1. Chemical structures of azithromycin (A) and roxithromycin (B).

2.2. LC–MS/MS instrument and conditions

Liquid chromatographic separation and mass spectrometric detection were performed using the Finnigan™ TSQ Quantum Discovery MAX™ LC–MS/MS system consisted of a Finnigan™ Surveyor LC pump, a Finnigan™ Surveyor autosampler and combined with a triple-quadrupole TSQ Quantum mass spectrometer (Thermo Electron Corporation). The chromatographic separation was on a SHISEIDO CAPCELL PAK C_{18} (3.0 mm × 75 mm, 3 μm) analytical column at 30 °C. The mobile phase consisted of 10 mM ammonium acetate (adjusted pH to 5.2 with0.1% acetic acid)–methanol (18:82, v/v) and was isocratically eluted at a flow rate of 0.3 mL/min.

The tandem MS system is equipped with an electro spray ionization (ESI) source, and run with the Xcalibur 1.1 software (Thermo Electron Corporation). With scan time of 0.10 s per transition, the mass spectrometer was operated in positive ion and selected reaction monitoring (SRM) mode with precursor-to-product qualifier transition m/z 375 $[M+2H]^{2+} \rightarrow$ 591 for azithromycin, and m/z 837 $[M+H]^{+} \rightarrow$ 679 for roxithromycin (IS). Spray voltage was optimized at 5000 V, transfer capillary temperature at 300 °C, sheath gas and auxiliary gas (nitrogen) pressure at 30 and 8 arbitrary unites (set by the LCQ software, Thermo Electron Corporation), respectively. Argon was used as collision gas at a pressure of 1.5 mTorr and collision energy was 35 V.

2.3. Tissue preparation

Rabbits were sacrificed by injecting pentobarbital sodium (100 mg/kg) into a marginal ear vein. Immediately after death, bulbar conjunctiva were excised, weighed, and homogenized with 1 mL phosphate buffered saline (PBS) in 5 mL polypropylene tubes using an Omni 2000 homogenizer (Omni International, Gainsville, Virginia). Tubes were then spun at 800 rpm for 5 min in a Sorvall RC3B refrigerated centrifuge to collapse the foam that formed during homogenization. Both the liquid and foam phase were then transferred into a 1.0 mL Eppendorf tube and spun at 14,000 rpm for 3 min. This process eliminated the remaining foam, forming a pellet of solid conjunctiva tissue. The liquid phase was used as blank conjunctiva samples.

2.4. Preparation of standards and quality control samples

Although azithromycin has poor water solubility, its solubility in buffered solutions increases with the decrease of pH. In order to satisfy the ocular physiology requirements, phosphate buffer (pH 6.0) was used as solvent. Standard stock solutions of azithromycin was prepared in phosphate buffer (pH 6.0) at the concentration of 200 μg/mL, which containing equivalent dose of sodium hyaluronate. Roxithromycin was prepared in acetonitrile at the concentration of 200 μg/mL. The internal standard solution was diluted with acetonitrile to 2000 ng/mL. The azithromycin solution was then serially diluted with acetonitrile to provide working standard solutions of desired concentrations. All the solutions were stored at −20 °C.

Calibration standards were prepared by spiking 0.2 mL of blank conjunctiva samples with working standard solutions of azithromycin. The effective concentrations in standard conjunctiva samples were 10, 20, 100, 200, 400, 800, 1600 and 2000 ng/mL. Each sample also contained 40 ng (20 μL × 2000 ng/mL) of the internal standard. In each run, a blank sample (no IS) was also analyzed. One calibration curve was constructed on each analysis day using freshly prepared calibration standards.

Quality control (QC) samples were prepared daily by spiking blank conjunctiva samples with proper volume of one of the calibration standards solution mentioned above to produce

a final concentration equivalent to 10 ng/mL (LLOQ), 20 ng/mL (low level), 400 ng/mL (middle level) and 1600 ng/mL (high level) of azithromycin. The following procedures were the same as described above.

2.5. Sample pretreatment

200 μL rabbit conjunctiva samples and 20 μL of internal standard (2000 ng/mL) were pipetted into a 1.5 mL Eppendorf tube. A 600 μL aliquot of acetonitrile was added to deproteinization. The sample was mixed vigorously for 3 min and centrifuged at 12,000 × *g* for 10 min. A 5 μL aliquot of the supernatant was injected into the LC–MS/MS system.

2.6. Method validation

2.6.1. Selectivity

Selectivity was studied by comparing chromatograms of six different batches of blank conjunctiva samples obtained from six subjects with those of corresponding standard conjunctiva samples spiked with azithromycin and roxithromycin.

2.6.2. Linearity and lower limit of quantification (LLOQ)

Calibration curves were prepared by assaying standard conjunctiva samples at nine concentrations of azithromycin ranging 10–2000 ng/mL. The linearity of each calibration curve was determined by plotting the nominal concentration (y) of azithromycin to the peak area ratio (x) of azithromycin versus roxithromycin (IS) The calibration curves were constructed by weighted ($1/x^2$) least square linear regression.

The lower limit of quantification is defined as the lowest concentration on the calibration curve, it was validated using an LLOQ sample for which an acceptable accuracy (%) ranged from 90% to 110% and a precision (RSD) below 10% were obtained.

2.6.3. Precision and accuracy

The intra-batch precision and accuracy was determined by analyzing five sets of spiked conjunctiva samples of azithromycin at each QC level (10, 20, 400 and 1600 ng/mL) in a batch. The inter-batch precision and accuracy was determined by analyzing five sets of spiked plasma samples of azithromycin at each QC level (10, 20, 400 and 1600 ng/mL) in three consecutive batches. The concentration of each sample was calculated using standard curve prepared and analyzed on the same day. The precision was expressed as the relative standard deviation (RSD). The accuracy of the method was evaluated by analysis the quality control samples spiked with standard solutions and expressed as a percentage error of measured concentrations versus nominal concentrations.

2.6.4. Recovery and matrix effect

The absolute recovery of azithromycin through the deproteinization procedures was determined at three concentrations (20, 400 and 1600 ng/mL). A known amount of azithromycin was added to blank conjunctiva samples prior to deproteinization as described in the section of "sample pretreatment", and then the IS (roxithromycin) was added after deproteinization to eliminate bias introduced by sample processing. According to the guidance of USFDA [24], recovery experiments should be performed at three concentrations (low, medium, and high). This procedure was repeated for five replicates at three concentrations of 20, 400 and 1600 ng/mL. The absolute recovery was calculated by comparing the peak area ratio of azithromycin/roxithromycin of deproteinized samples to the peak area ratio of azithromycin/roxithromycin of standards.

In order to evaluate the matrix effect (ME) on the ionization of analyte, i.e. the potential ion suppression or enhancement due to the matrix components. Azithromycin at three concentration levels were added to the deproteinization of 0.2 mL blank conjunctiva samples, dried and reconstituted with 200 μL of mobile phase, the corresponding peak areas (A) were compared with those of the azithromycin standard solutions dried directly and reconstituted with the same mobile phase (B). The ratio ($A/B \times 100$)% was used to evaluate the matrix effect. The matrix effect of internal standard was also evaluated using the same method.

2.6.5. Stability

The short-term stability of azithromycin was assessed by determining QC conjunctiva samples kept at room temperature for 24 h, which exceeded the routine preparation time of samples. The long-term stability was evaluated by determining QC conjunctiva samples kept at low temperature (−20 °C) for 10 days. The post-preparative stability was measured by determining QC samples kept under the auto-sampler conditions (room temperature) for 24 h. The freeze and thaw stability was tested by analyzing QC samples undergoing three freeze (−20 °C)–thaw (room temperature) cycles on consecutive days. The stock solution stability of azithromycin and the IS were evaluated by analyzing their working solutions kept at room temperature for 24 h, respectively. All the samples were analyzed and the experimental concentrations were compared with the nominal values.

3. Results and discussion

3.1. Selection of IS

For an LC–MS–MS analysis, utilization of stable isotope-labeled drugs as internal standards proves to be helpful when a significant matrix effect is possible. In our laboratory, isotope-labeled analyte was not obtainable to serve as IS, therefore, a compound being structurally or chemically similar to the analyte was considered. In LC–MS/MS the I.S. should also have similar chromatographic and mass spectrometric behaviours to the analyte, and mimic the analyte in any sample preparation steps. Roxithromycin was chosen as the internal standard for the assay because of its similarity of structure, retention time and ionization to azithromycin.

3.2. Chromatography and mass spectrometry

The separation and ionization of azithromycin and roxithromycin were affected by the composition of mobile phase. Therefore, the selection of mobile phase components was critical. Ammonium acetate was employed to supply the ionic strength. With buffers of lower strength, the peak shapes were not satisfactory, whereas with higher strength there was an improvement in the peak shapes. In this study, we also observed that increasing the concentration of ammonium acetate in mobile phase would prolong the retention time of analyte under the same organic solvent percentage.

A mixture of 10 mM ammonium acetate buffer–methanol was finally adopted as the mobile phase.

The molecule of azithromycin contains 2 nitrogen atoms, 1 locating the 15-membered ring, and the other attaching to the sugar. These two nitrogen atoms could be simultaneously protonated easily during ionization process in the acidic condition.

In ESI modes, the molecular ions with an m/z 375 $[M+2H]^{2+}$ and m/z 749 $[M+H]^{+}$ for azithromycin were produced (Fig. 2-1). Roxithromycin (IS) molecule was protonated to form molecule ion with m/z 837 $[M+H]^{+}$ because of one nitrogen in the molecule (Fig. 2-2). In the MS–MS spectra, the analysis of precursor-to-product ion pair was decided at the most sensitive mass transition which was from m/z 375 $[M+2H]^{2+}$ to m/z 591 as the precursor ion m/z 375 was more sensitive than m/z 749 for the azithromycin (Fig. 3-1),

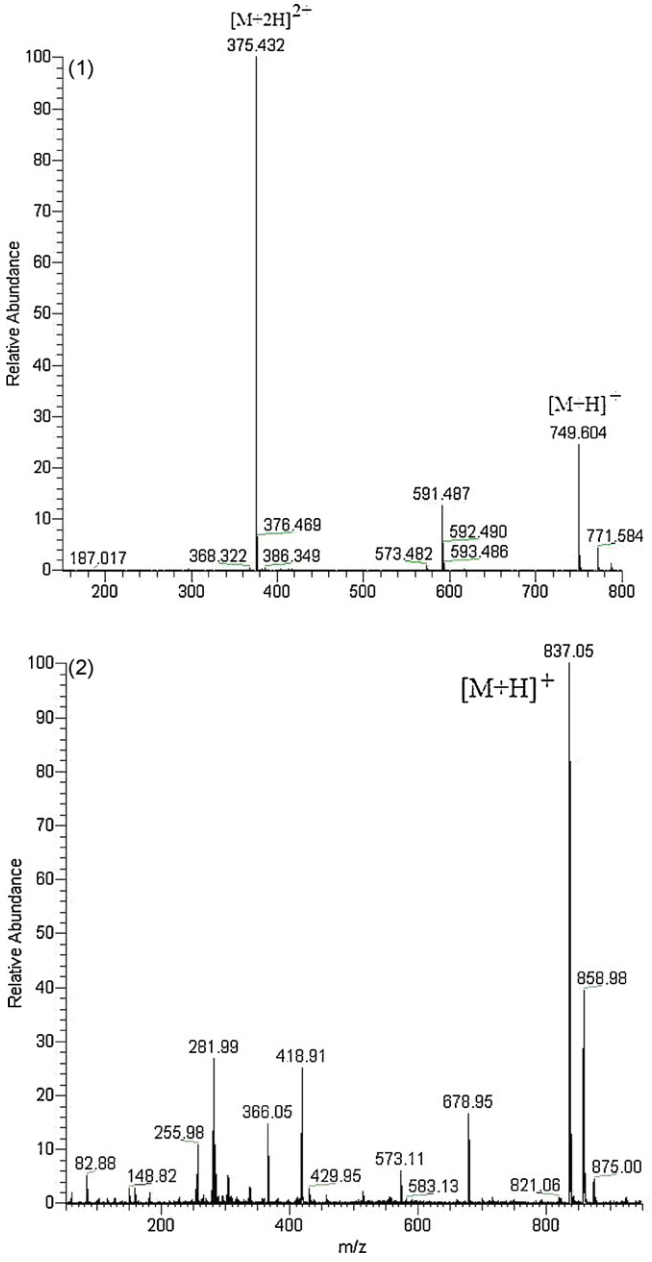

Fig. 2. Full scan ESI (+) precursor ion mass spectra of azithromycin (1) and IS (2).

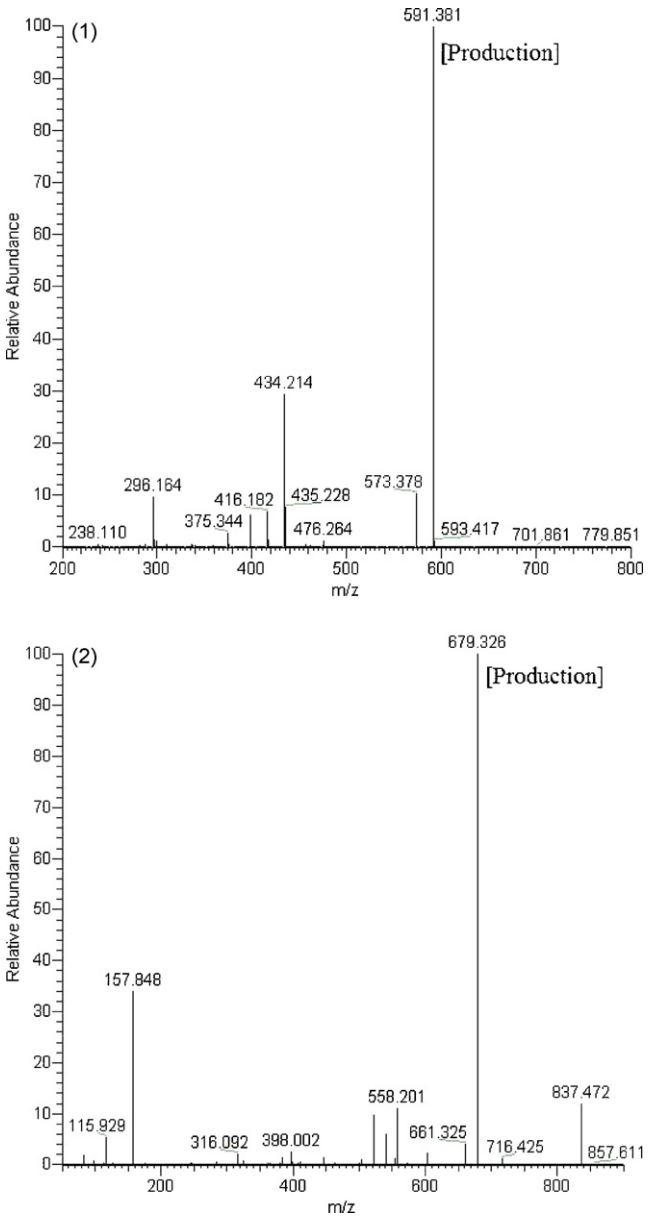

Fig. 3. Full scan ESI (+) product ion mass spectra of azithromycin (1) and IS (2).

and from *m*/*z* 837 to 679 for the IS (Fig. 3-2). Other main mass spectrometry parameters, such as spray voltage, capillary temperature, sheath gas and auxiliary gas pressure, source CID, collision gas pressure and collision energy, were also optimized. Finally, the transition ions of *m*/*z* 375 $[M+2H]^{2+} \rightarrow$ 591 for azithromycin, *m*/*z* 837 $[M+H]^{+} \rightarrow$ 679 for the IS were set as detecting ions for obtaining maximum sensitivity. The positive ion ESI mass spectrum and the MS/MS product ion spectrum of these compounds are shown in Figs. 2 and 3.

Two channels were used for recording the response, channel 1 for azithromycin with a retention time of 1.46 min and channel 2 for roxithromycin with a retention time of 2.37 min. As shown in Fig. 4, both azithromycin and roxithromycin were well separated with good peak shapes. Both azithromycin and roxithromycin were rapidly eluted with retention times less than 3 min, and the total run time was 3.0 min per sample. The short analysis time may meet the requirement for high sample throughput in bioanalysis.

3.3. Method validation

3.3.1. Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank conjunctiva sample with the corresponding spiked conjunctiva sample. As shown in Fig. 4-1, -2 and -3, no interference from endogenous substance was observed at the retention time of azithromycin and roxithromycin.

3.3.2. Linearity and LLOQ

The nine-point calibration curve was linear over the concentration range 10–2000 ng/mL. The calibration model was selected based on the analysis of the data by linear regression with/without intercepts and weighting factors ($1/x$, $1/x^2$ and 1). The best linear fit and least-squares residuals for the calibration curve were achieved with a $1/x^2$ weighing factor, giving a mean linear regression equation for the calibration curve of: $y = 842.15x + 5.8211$ ($r = 0.9998$).

The lower limit of quantification for azithromycin was 10 ng/mL with 5 μL injected onto the LC–MS/MS systems with precision and

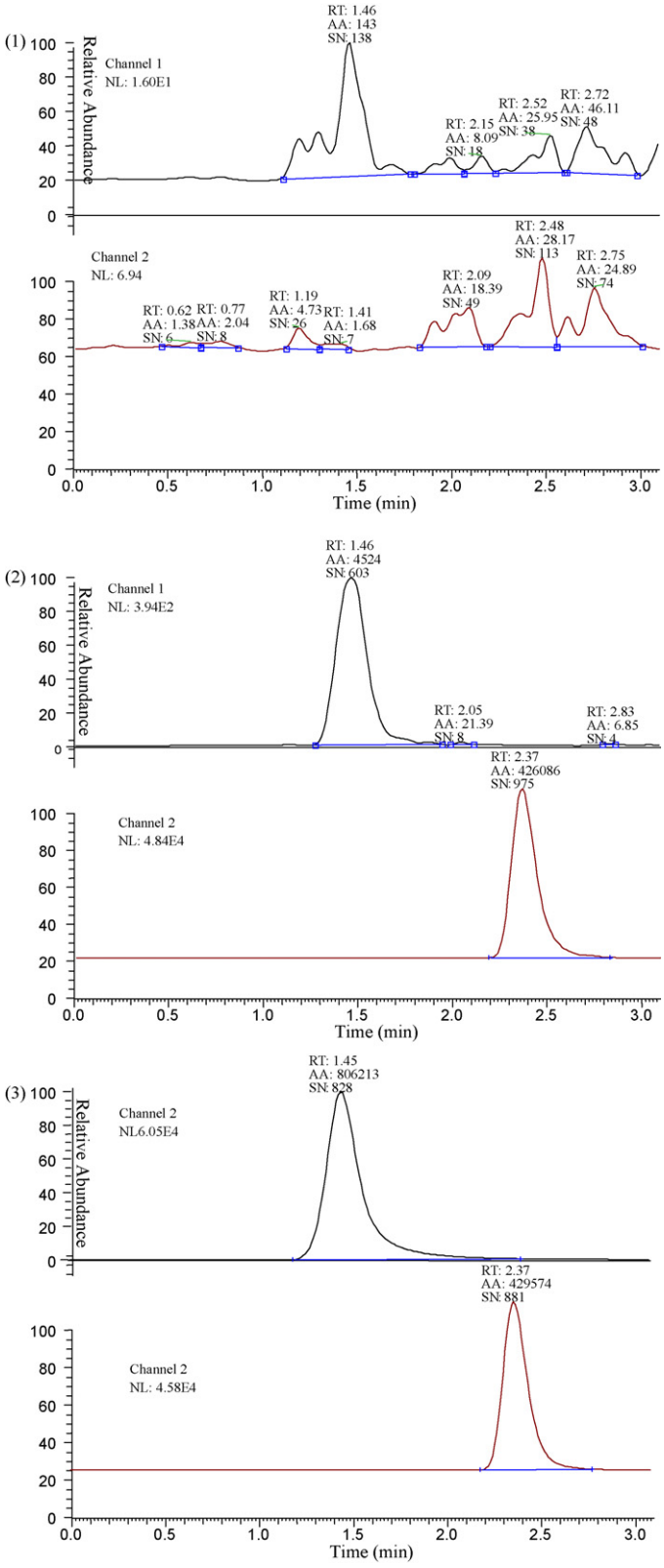

Fig. 4. Representative SRM chromatograms for azithromycin and the IS resulting from analysis of blank conjunctiva (drug and IS free, 1); a blank conjunctiva sample spiked with azithromycin at the LLOQ of 10 ng/mL and roxithromycin (200 ng/mL, 2); a blank conjunctiva sample spiked with azithromycin at 1600 ng/mL and roxithromycin at (200 ng/mL, 3). *Note*: the vertical axis represents relative abundance normalized to the indicated value (NL; note the difference between 1, 2 and 3); RT represents retention time; AA represents area; SN represents signal-to-noise.

Table 1
Precision and accuracy for determination of azithromycin in conjunctiva samples (intra-day: $n = 5$; inter-day: $n = 5$; $\bar{X} \pm$ SD).

Added (ng/mL)	Measured (ng/mL)	Intra-day RSD (%)	Inter-day RSD (%)	Accuracy (%)
10 (LLOQ)	9.72 ± 0.57	6.83	5.57	97.2
20 (low)	18.4 ± 1.64	4.30	3.02	92.0
400 (middle)	414.7 ± 4.04	7.38	6.55	103.7
1600 (high)	1590.4 ± 32.6	5.88	8.59	99.4

Table 2
Stability of azithromycin in conjunctiva samples at three QC levels ($n = 5$).

Stability	Accuracy ($\bar{X} \pm$ SD) (%)		
	20 (ng/mL)	400 (ng/mL)	1600 (ng/mL)
Short-term stability	103.7 ± 2.5	96.4 ± 3.9	95.0 ± 1.7
Long-term stability	98.3 ± 6.8	92.9 ± 2.6	98.1 ± 3.4
Freeze–thaw stability	101.5 ± 1.3	104.3 ± 2.3	99.4 ± 4.1
Post-preparative stability	99.7 ± 4.2	96.1 ± 3.7	102.8 ± 3.6

accuracy presented in Table 1 with RSD lower than 10%. A corresponding chromatogram is given in Fig. 4-2.

3.3.3. Precision and accuracy

The data of intra-day and inter-day precision and accuracy for the method are listed in Table 1. The intra-day precision for low, mid and high QC levels of azithromycin were 4.30%, 7.38% and 5.88%, respectively, and that of inter-day analysis were 3.02%, 6.55%, 8.59%, respectively, with an accuracy ranged from 92% to 105%. The precision and accuracy of the present method conform to the criteria for the analysis of biological samples according to the guidance of USFDA [23]. The results of the precision and accuracy of the proposed method were acceptable for bioequivalence.

3.3.4. Recovery and matrix effect

The extraction recoveries of azithromycin from blank conjunctiva were 96.0 ± 5.8%, 101.3 ± 3.7%, and 93.4 ± 6.5% at concentration levels of 20, 400 and 1600 ng/mL, respectively, and the mean extraction recovery of roxithromycin was 96.6 ± 4.7%.

In terms of matrix effect, all the ratios $(A/B \times 100)$% defined as in Section 2 were between 93% and 110%, which means no matrix effect for azithromycin and roxithromycin in this method.

3.3.5. Stability

The stock solution of azithromycin and roxithromycin were found to be stable at room temperature for 4 h and at 4 °C for 25 days. The results from all stability tests presented in Table 2 demonstrated a good stability of azithromycin over all steps of the determination. The method is therefore proved to be applicable for routine analysis.

4. Conclusion

In summary, a sensitive, selective and rapid method with an LLOQ of 10 ng/mL which is far below the MIC demanded in curing conjunctivitis, is described for the quantification of azithromycin from conjunctiva by LC–MS/MS in positive ionization mode using selected reaction monitoring. Unlike the published complicated sample pretreatment methods for extracting drugs from the biosamples using the organic solvents (i.e. diethyl ether, methanol, methyl tert-butyl ether, hexane, etc.), deproteinization procedures is just one step which can curtail test's time that is important for large sample batches. It was shown that this method is suitable for the analysis of azithromycin in conjunctiva samples collected for pharmacokinetic, bioavailability or bioequivalence studies.

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