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Quantification of aesculin in rabbit plasma and ocular tissues by high performance liquid chromatography using fluorescent detection: Application to a pharmacokinetic study

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ABSTRACT

A simple and sensitive high performance liquid chromatography method with fluorescence detection (HPLC-FD) was described for the determination of aesculin (AL) at low concentrations in rabbit plasma and ocular tissues. After deproteinization by methanol using pazufloxacin mesilate (PM) as an internal standard (I.S.), supernatants were evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in mobile phase and a volume of 20 μL was injected into the HPLC for analysis. Analytes were separated on an Ultimate XB-C18 column (250 mm × 4.6 mm i.d., 5 μm particle size) and protected by a ODS guard column (10 mm × 4.0 mm i.d., 5 μm particle size), using acetonitrile–0.1% triethylamine in water (adjusted to pH 3.0 using phosphoric acid) (12:88, v/v) as mobile phase with a flow rate of 1.0 mL/min. The wavelengths of fluorescence detector (FD) were set at 344 nm for excitation and 466 nm for emission. The lower limit of quantitation (LOQ) for AL was 0.80 ng/mL for plasma and vitreous body, 1.59 ng/mL for aqueous humor, and 6.55 ng/g for iris and 1.66 ng/g for retina. The method was used in the study of AL concentrations in plasma and ocular tissues after topical administration of AL eye drops.

1. Introduction

AL (aesculin) is a coumarin derivative contained in many plants such as dry tegument of *Aesculus hippocastanum* Linn. and the dry tegument of different categories of *Fraxini chinensis* Roxb. [1–3]. It has multiple biological activities such as anti-oxidation, anti-inflammation, vision improvement and xanthine oxidase inhibition [4–8]. Eye drops containing AL are used for the treatment of eye diseases, such as conjunctivitis and age-related macular degeneration [5,9,10]. AL has been evaluated in clinical studies for its microcirculation improving effects in the posterior segment of the eye [9].

Eye drops are routinely used in ophthalmology for the treatment of ocular diseases. However, as only small amount of the drug substance delivered by eye drop can reach the posterior segment, the posterior segment drug level is relatively low [11–13]. Fortunately, many of the pharmacological effects of AL are exerted at low nanomolar concentrations [5,14]. Quantification of AL at low concentrations following topical administration in plasma and ocular tissues especially retina requires selective and sensitive bioanalytical techniques.

Many methods, including high performance liquid chromatography [5], high performance capillary electrophoresis [1,15], capillary electrophoresis end-column amperometric detection [16], capillary zone electrophoresis [1] and non-aqueous capillary electrophoresis with UV detection [17,18] have been developed to analyze AL. Fluorospectrophotometric, ultraviolet and mass spectrometric detectors are currently used to quantify AL [14,19,20]. The major shortcoming of fluorospectrophotometry is the endogenous interference, could not be separated with the AL and I.S. in biological samples. HPLC-UV with a LOQ of 57.4 ng/mL and LC–ESI-MS with a LOQ of 12.5 ng/mL failed to detect the extremely low concentration of AL in posterior intraocular tissues directly. Up to date, the ocular disposition profiles of AL have not been reported.

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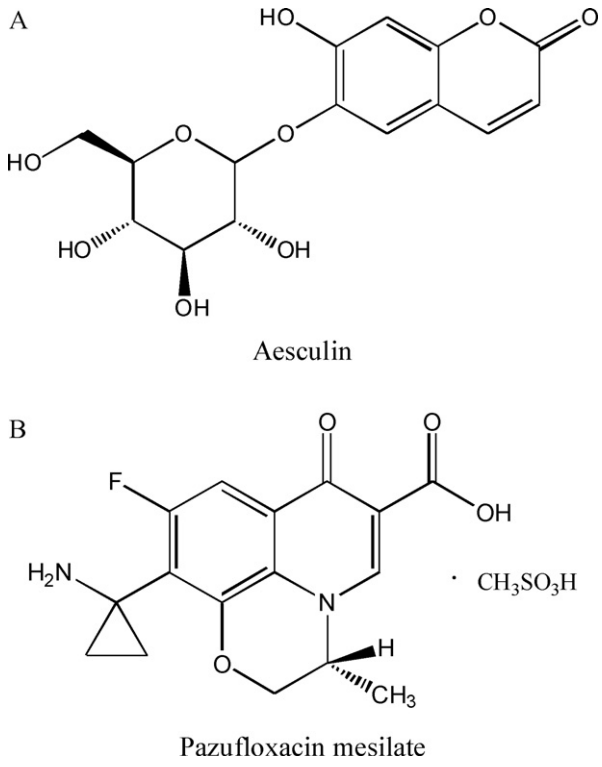

Fig. 1. Structure of AL (A) and pazufloxacin mesilate (B).

Therefore, it is of interest to develop a sensitive and reliable method to measure AL in ocular issues.

Since AL has fluorophoric properties, fluorescent detection (FD) was expected to provide an inexpensive, sensitive, and specific detection of AL in biological samples. In this study, a sensitive and reliable HPLC-FD method was developed and validated to quantify AL in rabbit plasma and ocular tissues. The ocular disposition profiles of AL was subsequently assessed in rabbits with this HPLC-FD method. To the authors' knowledge, this is the first report on the ocular pharmacokinetics of AL.

2. Materials and methods

2.1. Materials and reagents

AL ($C_{15}H_{16}O_9$; MW = 340.28, purity > 99.5%) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Pazufloxacin mesilate (PM, I.S., $C_{17}H_{19}FN_2O_7S$; MW = 414.41, purity > 99.0%) was purchased from Jiangsu Yabang Pharmaceutical & Chemical Co., Ltd. (Jiangsu, China). The molecular structure of AL and PM were illustrated in Fig. 1. HPLC grade methanol and acetonitrile were obtained from Fisher Scientific International Inc. (Fairlawn, NJ, USA). Analytical grade orthophosphoric acid (H_3PO_4) and triethylamine were supplied from Chengdu Fang Zhou Sci. and Tech. Co., Ltd. (Chengdu, China). All chemicals and solvents were of analytical or HPLC grade. All standard solutions and mobile phases were prepared using glass-double distilled water and passed through a 0.22 μm membrane filter before use.

2.2. Sample preparation

Healthy adult New Zealand albino rabbits (2.5–3 kg) were obtained from the Experimental Animal Center of Sichuan University, Sichuan, China. After rabbits were anaesthetized with an injection of sodium pentobarbital (30 mg/kg) into one auricular vein, blood was rapidly collected in heparinized polypropylene tubes from the other auricular vein. The blank plasma was separated by immediately centrifuging at 3000 rpm for 10 min. Then animals were euthanized under anesthesia. The eyes were enucleated and manually dissected to isolate and harvest individual tissues, including aqueous humor, iris, vitreous body and retina. Then iris and retina were weighed and homogenized with 1 mL phosphate buffered saline (PBS) in 5 mL polypropylene tubes using an Omni 2000 homogenizer (Omni International, Gainesville, Virginia). Aqueous humor and vitreous body samples were processed without any further dilution. All the samples were stored at −20 °C until required.

Stock solutions of AL and I.S. were prepared by dissolving accurately weighed AL and PM in methanol to yield final concentrations of 1.01 mg/mL and 12 μg/mL, respectively. The stock solutions were stored in volumetric flasks sealed with laboratory parafilm sealing film at −20 °C (stable for at least 2 months) and brought to 4 °C before use [19,21]. The standard solution was diluted, respectively, with blank plasma, aqueous humor, vitreous body and treated blank tissue sample to yield the calibration standard solutions of 0.8, 1.59, 3.19, 6.37, 12.75, 25.5 and 51 ng/mL. Quality control (QC) samples were prepared to the target concentrations which were, respectively, selected to cover the range of the calibration curve in the same way as the samples for calibration. The QC samples were then divided into 100 μL aliquots in tightly closed microtubes and kept frozen at −20 °C until required.

The solution of PM was stored at −20 °C and brought to 4 °C before use. To each of disposable plastic tubes with 100 μL biological samples (including samples for calibration, QC and pharmacokinetic study), 2 μL of I.S. (PM, 12 μg/mL) was added except the blank biological sample at approximately 4 °C using a 2–20 μL pipette (Eppendorf AG, Germany). After vortexed for 5 min on a vortex mixer (Jiangxi Medical Instrument Group, China), 500 μL of methanol was added to each of these samples. The obtained solution was vortexed for 10 min at room temperature. The mixture was centrifuged at 12,000 rpm for 20 min to remove any protein and the supernatant was removed into 1.5 mL glass tubes and evaporation was completed to dryness under nitrogen at 40 °C. After adding 100 μL of the mobile phase, vortexing (5 min) and centrifuging (12,000 rpm for 20 min), a volume of 20 μL of the supernatant was injected into the HPLC for analysis.

2.3. High-performance liquid chromatographic apparatus and chromatographic condition

The HPLC system was Shimadzu LC-10A series (Shimadzu Corp., Kyoto, Japan), consisting of two LC-10AVP pumps, a vacuum degasser, a thermostated column compartment, a RF-10AXL fluorescence detector (FD) system and a manual sample valve injector with a 50 μL loop. The analytical column employed was an Ultimate C18 column (250 mm × 4.6 mm i.d., 5 μm particle size; Welch Materials, Ellicott, MD, USA) and protected by an ODS guard column (10 mm × 4.0 mm i.d., 5 μm particle size). For robustness evaluation, the HPLC system of Waters 2690 with a waters-474 fluorescence detector (FD) system was used (Waters Corp., USA). And different columns were used including a Kromasil C18 column (250 mm × 4.6 mm i.d., 5 μm particle size; Akzo-Nobel Corp., Amsterdam, Netherlands) and a Diamonsil C18 column (250 mm × 4.6 mm i.d., 5 μm particle size; Dikma Technologies Inc., USA). Fluorescence detector excitation and emission wavelength were set at 344 nm and 460 nm, respectively. The mobile phase comprised of acetonitrile–0.1% triethylamine in water (adjusted to pH 3.0 using phosphoric acid) (12:88, v/v), was filtered through a 0.22 μm cellulose membrane filter (Auto Science, Tianjin, China). The mobile phase was then pumped through the system at a rate of 1.0 mL/min. Twenty microlitres of sample solution was then

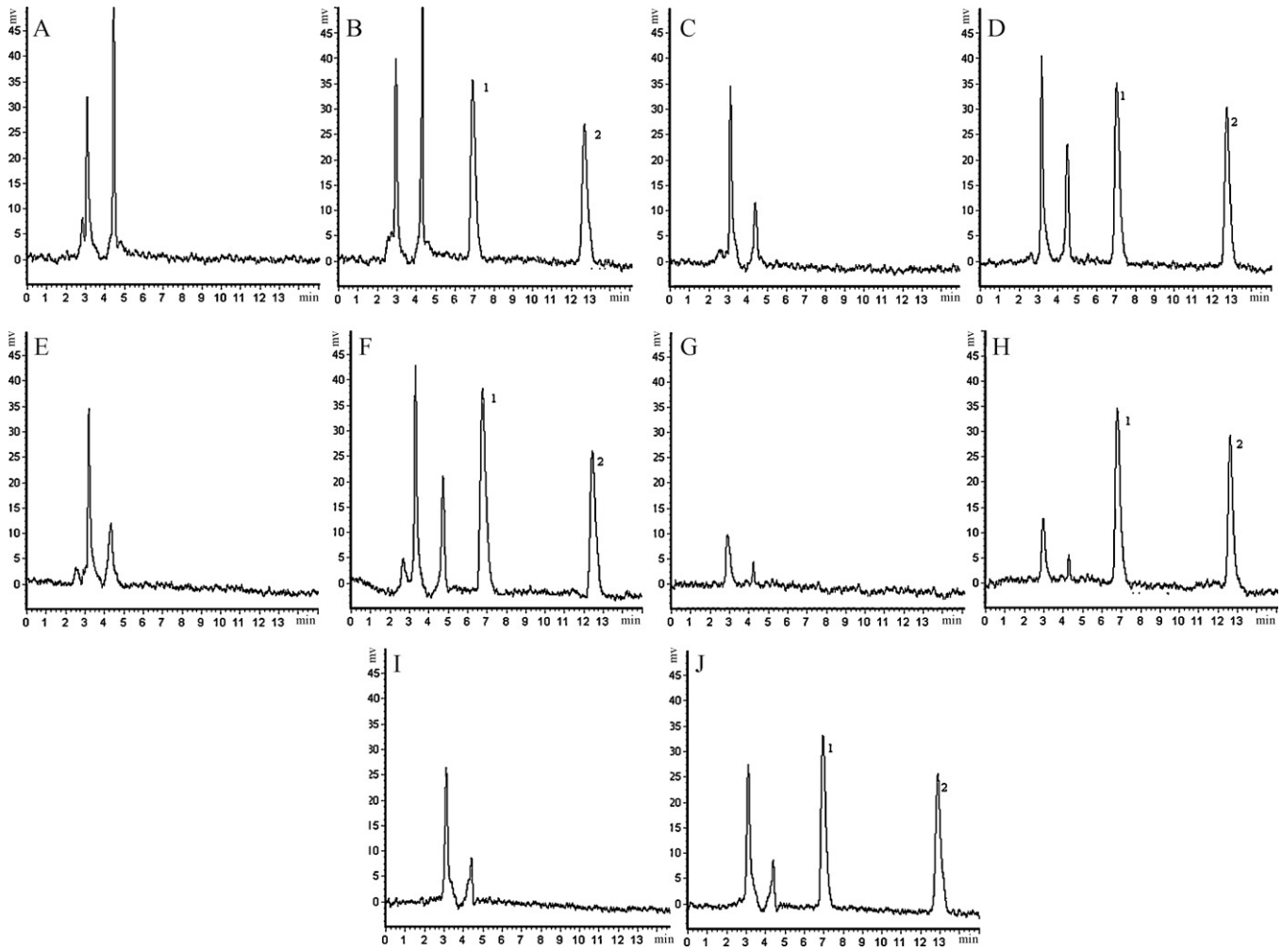

Fig. 2. Representative chromatograms of AL. (A) Blank plasma. (B)Plasma spiked with AL (6.38 ng/mL) and I.S. (PM, 240 ng/mL). (C) Blank iris. (D) Iris spiked with AL (6.38 ng/mL) and I.S. (PM, 240 ng/mL). (E) Blank retina. (F) Retina spiked with AL (6.38 ng/mL) and I.S. (PM, 240 ng/mL). (G) Blank aqueous humor. (H) Aqueous humor spiked with AL (6.38 ng/mL) and I.S. (PM, 240 ng/mL). (I) Blank vitreous body and (J) vitreous body spiked with AL (6.38 ng/mL) and I.S. (PM, 240 ng/mL). Peak 1: AL; Peak 2: PM.

injected onto the column. Chromatography was performed at 30 °C using a 15-min run time. All chromatograms obtained were evaluated by the peak area ratio of AL to the I.S. The calibration curve was plotted with the peak area ratio of AL to the I.S. against the concentration of AL in biological samples. The data demonstrated a linear function for AL according to the equation:

$$Y = aX + b(r^2)$$

where Y is the peak area ratio of AL to the I.S., X is the plasma concentration of AL (ng/mL), a is the slope and b is the intercept, and r^2 is the correlation coefficient.

2.4. Validation of the method

For method validation and linearity studies, blank biological samples obtained from healthy adult New Zealand albino rabbits (2.5–3 kg) were used. The specificity of the assay was evaluated by comparing the blank biological samples with biological samples spiked with AL and the I.S. The efficiency of the extraction procedure was observed at three different concentrations for different biological samples. Recovery was calculated by comparing the respective peak areas of the chromatograms of the extracted samples with the untreated standards solution containing an equivalent amount of the compounds. Calibration curves were constructed by linear least-squares regression analysis plotting of peak-area ratio (AL/I.S.) versus the drug concentrations. The LOQ was determined as the lowest concentration with a signal-to-noise ratio of 10. The LOD in biological sample was calculated as the amount of the injected sample, which resulted in a signal-to-noise ratio of 3. The accuracy and precision of the method were evaluated with QC samples for all above-involved biomatrices on three consecutive days, accompanied by a standard calibration curve on each analytical run. These concentrations were selected to cover the range of each the calibration curve, respectively.

The quality control (QC) samples were assayed under several different conditions to assess the stability of AL in rabbit biological samples. One set of QC samples was stored at room temperature (approximately 22–25 °C) for 24 h in volumetric flask. The stability of the sample at room temperature was evaluated by comparing the assay results of the stored QC samples with that of the freshly thawed QC samples. Another set of QC samples was subjected to three freeze–thaw cycles and was then assayed to evaluate freeze–thaw stability of AL in biomatrices mentioned above. Long-term stability was studied by assaying samples that had been stored at −20 °C for a certain period of time (0, 5, 10, 30, 45 and 60 days). AL was considered stable under storage conditions if the assay percent recovery was found to be 90–110% of the nominal initial concentration.

To determine the recovery of AL in rabbit plasma and ocular matrices, an aliquot of blank biomatrices was spiked with AL to

Table 1
Equations of calibration curves for the analysis of AL in plasma and ocular tissues ($n = 3$).

Sample	Concentrations (ng/mL)	Equation[a]	Correlation (r^2)
Plasma	0.80–51.0	$y = 0.267x - 0.067$	0.9988
Iris	0.80–51.0	$y = 0.126x + 0.024$	0.9992
Retina	0.80–25.5	$y = 0.064x - 0.062$	0.9989
Aqueous humor	1.59–51.0	$y = 0.040x + 0.110$	0.9991
Vitreous humor	0.80–51.0	$y = 0.366x - 0.021$	0.9999

[a] Y is the peak area ratio of AL to the I.S.; X is the plasma concentration of AL (ng/mL).

achieve a final target concentration. The absolute recoveries of AL were determined by comparing peak areas of extracted QC samples with those of corresponding concentrations standard solutions. The analysis was performed for three replicates at the target concentration levels.

2.5. Dilution test

In order to determine the feasibility of the method to analyze samples whose concentrations extend beyond the upper limit of the calibration range, AL samples in plasma and ocular matrices prepared at a nominal concentration of 127.5 ng/mL ($n = 5$) were diluted 10-fold using blank matrix. The diluted samples were analyzed and the mean concentrations were compared to the nominal value after the dilution factor was applied.

2.6. Application of the method

All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals in Sichuan University, Chengdu, China, and conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Adult New Zealand albino rabbits (2.5–3 kg) were used in the study. A single dose of 100 μL of formulation containing 1 mg/mL AL (AL water solution prepared by our team) was placed in the culde-sac of both eyes of all animals. The rabbits were anesthetized at collection times after dosing. The biological sample was collected according the method described in Section 2.2 and stored at −20 °C until prepared for HPLC analysis. Pharmacokinetics parameters were calculated using DAS (drug and statistics) software (developed by Sun Ruiyuan professor, Institute of Materia Medica, Wannan Medical College, Wuhu, Anhui 241001, China).

Table 2a
Assay precision and accuracy of AL in rabbit plasma and ocular tissues (intra-day: $n^a = 6$; inter-day: $n^a = 6$; $X \pm$ S.D.).

Sample	AL nominal concentration (ng/mL)	AL measured concentration (ng/mL)	R.S.D. (%)[b]	RR (%)[c]
Plasma	Inter-day assay			
	0.80	0.86 ± 0.02	2.15	108.4
	6.37	6.47 ± 0.09	1.44	101.6
	51.0	52.5 ± 0.17	0.32	103.0
	Intra-day assay			
	0.80	0.85 ± 0.02	2.01	106.1
	6.37	5.90 ± 0.08	1.33	92.7
	51.0	53.3 ± 0.20	0.38	104.6
Aqueous humor	Inter-day assay			
	1.59	1.47 ± 0.23	15.40	92.0
	6.37	6.38 ± 0.28	4.38	100.2
	51.0	51.6 ± 0.76	1.47	101.2
	Intra-day assay			
	1.59	1.56 ± 0.23	14.40	98.1
	6.37	6.82 ± 0.33	4.78	107.2
	51.0	49.1 ± 0.66	1.34	96.2
Vitreous body	Inter-day assay			
	0.80	0.75 ± 0.05	6.67	94.1
	6.37	6.54 ± 0.03	0.52	102.8
	51.0	50.0 ± 1.38	2.76	98.0
	Intra-day assay			
	0.80	0.73 ± 0.05	6.35	91.6
	6.37	6.99 ± 0.05	0.66	109.8
	51.0	47.4 ± 1.23	2.59	92.9

[a] Number of tissue samples extracted.
[b] R.S.D. = relative standard deviation.
[c] RR = relative recovery.

3. Results and discussion

3.1. Specificity

No endogenous interference was found at the retention times of AL and the I.S. This method was observed to be highly selective for the quantitation of AL in plasma and all ocular tissues with no interfering peaks from the constitutive matrix. Representative chromatograms for blank rabbit biological samples (plasma and ocular tissues) and rabbit biological samples spiked with AL (6.38 ng/mL) and the I.S. (240.0 ng/mL) are shown in Fig. 2, respectively. The I.S. and AL were well resolved with respective retention times of 7.04 min and 12.86 min.

Table 2b
Assay precision and accuracy of AL in rabbit ocular tissues (intra-day: $n^a = 6$; inter-day: $n^a = 6$; $X \pm$ S.D.).

Sample	AL nominal concentration (ng/mL)	AL measured concentration (ng/mL)	R.S.D. (%)[b]	RR (%)[c]
Iris	Inter-day assay			
	0.80	0.75 ± 0.003	0.46	94.6
	6.37	6.47 ± 0.09	1.45	101.5
	51.0	51.5 ± 0.49	0.95	101.0
	Intra-day assay			
	0.80	0.76 ± 0.01	1.38	95.0
	6.37	6.33 ± 0.07	1.05	99.5
	51.0	53.0 ± 0.61	1.15	104.0
Retina	Inter-day assay			
	0.80	0.89 ± 0.05	5.59	111.3
	3.19	3.40 ± 0.16	4.61	106.7
	25.5	25.8 ± 0.75	2.90	101.2
	Intra-day assay			
	0.80	0.84 ± 0.05	6.37	105.0
	3.19	3.27 ± 0.18	5.46	102.7
	25.5	27.1 ± 0.95	3.50	106.1

[a] Number of tissue samples extracted.
[b] R.S.D. = relative standard deviation.
[c] RR = relative recovery.

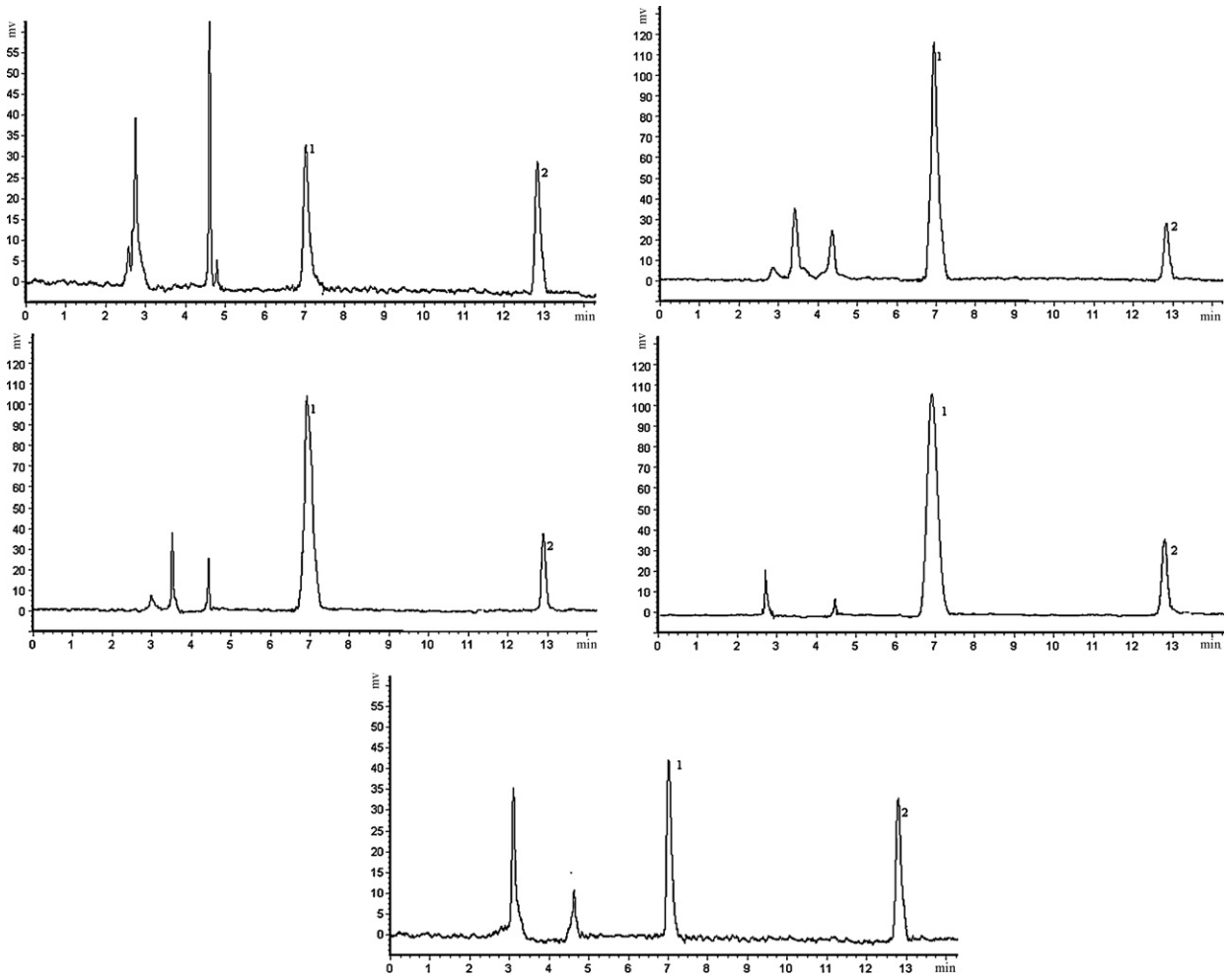

Fig. 3. Representative chromatograms of plasma and ocular tissues obtained at 60 min after a single topical administration in rabbits. (A) Plasma, (B) iris, (C) retina, (D) aqueous humor, and (E) vitreous body. The biological samples including aqueous humor and iris were diluted to appropriate concentration. Peak 1: AL; Peak 2: PM.

3.2. Linearity, limit of detection, precision and accuracy

The calibration curve was linear in the range from 0.80 to 25.5 ng/mL in retina, 1.59 to 51.0 ng/mL in aqueous humor, and the curves were linear over the concentration range from 0.8 to 51.0 ng/mL in plasma, iris and vitreous body. The calibration standards in a single analytical run were analyzed in duplicate for confirmation. The correlation coefficient (r^2) for each matrix was >0.99 when evaluated by weighted ($1/x^2$) linear regression. The linear regression equations were summarized in Table 1.

Based on the range of the calibration curve, the limit of quantitation (LOQ) was 1.59 ng/mL for aqueous humor and 0.8 ng/mL for other biomatrices. The LOQ for AL in this assay (0.8 ng/mL) represents a 75-fold increase in sensitivity over HPLC-UV assay (λ = 338 nm) for the measurement of AL levels in biomatrices that we have previously reported [19]. For tissue samples, the final results were normalized to individual tissue weights. The LOQ for iris and retina upon correcting for the dilution and normalization to tissue weights were 6.55 and 3.32 ng/g, respectively.

The LOD was 0.13 ng/mL for all biomatrices, determined as the concentration with signal-to-noise ratio of 3. The result of inter-day and intra-day precision (as relative standard deviation (R.S.D.)) and accuracy (as relative recovery (RR%)) of the QC samples for AL in rabbit plasma and ocular matrices were presented in Tables 2a and 2b. The intra-day and the inter-day relative standard deviation (R.S.D.) for all matrix were <15.5%. The intra-day and inter-day RR% for AL in all matrices were within 85–115% of their nominal values at all concentrations analyzed. Together, the available data demonstrate that the method is accurate and reproducible for the quantitation of AL in plasma and ocular tissues.

3.3. Recovery

The mean recovery for each matrix of QC samples was >85%. Using the same method, the recovery of I.S. in biomatrices was 99.7%. The mean result of recoveries for AL in rabbit plasma and ocular matrices were presented in Table 3.

3.4. Sample stability

Stock solution of AL in methanol was found to be stable for at least 2 months when stored at −20 °C. The stability of AL in QC samples was not less than 10 days and AL was stable in biological samples mentioned above for at least 7 days when stored frozen at −20 °C. (R.E. < −2.52%). Extracted biological samples were found to be stable for at least 24 h when the samples were kept at room temperature (22–25 °C) and the final concentrations were 92.3–100.5% of the initial values. AL was also stable following three freeze–thaw cycles. AL extracted from biological samples in the mobile phase was stable with relative errors less than −4.5% for at least 24 h at room temperature.

Table 3
The extraction recovery of AL in plasma and ocular tissues(n^a = 9).

Sample	AL concentration (ng/mL)	Mean recovery (%)
Plasma	0.80	110.5 ± 2.3
	6.37	97.6 ± 6.9
	51.0	92.3 ± 4.6
Iiris	0.80	102.2 ± 1.5
	6.37	94.7 ± 2.1
	51.0	97.4 ± 6.6
Retina	0.80	92.9 ± 2.9
	3.19	92.5 ± 2.7
	25.50	90.4 ± 4.2
Aqueous humor	1.59	90.2 ± 4.6
	6.37	92.5 ± 5.1
	51.0	96.3 ± 6.9
Vitreous body	0.80	91.2 ± 5.0
	6.37	98.8 ± 4.5
	51.0	95.3 ± 6.1

[a] Number of tissue samples extracted.

3.5. Dilution test

The measured AL concentrations in plasma and ocular tissue/fluid samples following a 10-fold dilution of the 127.5 ng/mL sample in corresponding plasma and ocular tissue were within 15% of their nominal values (data not shown). Clearly, this method could be used to quantify the biological samples with AL concentration exceeding the calibration range.

3.6. Robustness of method

This method showed that the chromatographic patterns were not significantly changed when different solvent sources and a different HPLC system (Waters, USA) were used. This method allowed variation in analytical parameters such as pH adjusted to 2.4–3.6, acetonitrile content in the mobile phase varied between 15% and 8%, and column temperature adjusted to 20–40 °C. The resolution factor between PM and AL was in the range of 12.0–24.9, and the retention time of AL and PM was in the range of 5.4–18.2 min, and they varied when the acetonitrile content in the mobile phase was changed. The peak area ratio of AL to PM for the same solution was not changed.

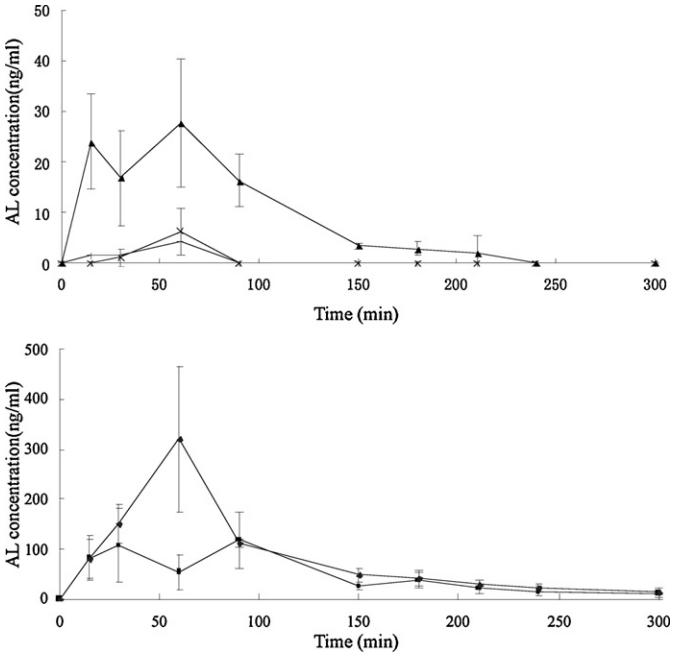

Fig. 4. Pharmacokinetic profiles of AL in rabbits after single ocular administration. Each point represented the mean of the concentration obtained from six rabbits. (*) Vitreous body; (▲) retina; (■) iris; (+) plasma; (⧫) aqueous humor.

Table 4
Pharmacokinetic parameters of AL in rabbits after single ocular administration (n^a = 6).

Parameter	Result (mean ± S.D.)			
	Plasma	Aqueous humor	Retina	Iris
AUC(0–t) (mg/L min)	223	24,901	2549	15,146
MRT(0–t)	39.8	91.2	67.8	113.1
$t1/2z$ (min)	72.1	102.7	48.7	52.6
T_{max} (min)	30	52.5	48.8	37.5
Vz/F (L/kg)	2.2	0.2	1.0	0.2
CLz/F (L/min/kg)	0.019	0.002	0.015	0.003
C_{max} (ng/mL)	4.4	344.3	33.9	168.8

[a] Number of animals studied.

3.7. Application of the method

The HPLC-FD method was subsequently applied in a pharmacokinetic study carried out in rabbits after ocular administration of AL eye drops. Typical chromatograms of biological samples obtained from pharmacokinetic study were shown in Fig. 3. The plasma/ocular tissue AL concentration–time profiles are shown in Fig. 4. The major pharmacokinetic parameters of AL are shown in Table 4. Clearly, AL had some exposure to posterior segment of the rabbit eyes after single eye drop administration.

4. Conclusion

In conclusion, a sensitive and reliable reversed-phase HPLC-FD method has been developed and validated to quantify AL in rabbit plasma and ocular tissues. This HPLC method was subsequently applied in a pharmacokinetic study carried out in rabbits after ocular administration of AL eye drops. AL was found to have some exposure to posterior segment of the eyes upon single ocular administration of AL eye drop. This HPLC method would facilitate future investigation on the ocular delivery of AL.

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