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Determination of tacrolimus in rabbit aqueous humor by liquid chromatography–electrospray ionization tandem mass spectrometry

Jin Yuan [a], Jia-qi Chen [a], Zhi-yong Xie [b], Jia-jie Zhai [b], Shi-you Zhou [a,*]

[a] Zhongshan Ophthalmic Center, Sun Yat-sen University, 54 South Xianlie Road, The State Key Laboratory of Ophthalmology, Guangzhou, Guangdong Province 510060, China
[b] Laboratory of Pharmaceutical Analysis & Quality Assessment, School of Pharmaceutical Sciences, Sun Yat-sen University, 74 Zhongshan Road II, Guangzhou 510080, China

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ABSTRACT

A simple, sensitive and selective liquid chromatographic–tandem mass spectrometric (LC–MS/MS) method was developed for the determination of tacrolimus (FK506) in rabbit aqueous humor. After a simple protein-precipitation by methanol, the post-treatment samples were separated on a reversed-phase, Thermo-Hypersil-BDS-C18 column with a mobile phase of a mixture of 0.1% formic acid in water, methanol and acetonitrile (5:85:10, v/v/v). Tacrolimus and ritonavir (internal standard, IS) were all detected by the selected reaction-monitoring (SRM) mode. The method developed was validated in rabbit aqueous humor with a daily working range of 0.5–100 ng/ml with correlation coefficient, $r > 0.99$ and a sensitivity of 0.5 ng/ml as lower limit of quantification, respectively. This method was fully validated for the accuracy, precision, possible matrix effect and stability. The method proved to be accurate and specific, and was applied to the pharmacokinetic study of tacrolimus in rabbit aqueous humor.

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1. Introduction

Tacrolimus (FK 506) is widely used after organ transplantation as a calcineurin inhibitor immunosuppressant. It has a mechanism of action similar to that of cyclosporine A, but is more potent [1]. It acts by binding to the immunophilin, then forms a calcineurin–calmodulin–calcium complex which inhibits calcium-phosphatase activity. This leads to blockage of the transcriptional activation of cytokines such as interleukin 2 (IL-2) which is critical in T-cell activation and of transcription factors such as the nuclear factor of activated T cells (NFAT) [2].

The use of FK506 is of special interest in ophthalmology because it was proved to be effective in the treatment of immune-mediated diseases such as corneal graft rejection [3], limbal allograft rejection [3,4], keratoconjunctivitis [5], scleritis [6], cicatricial pemphigoid [7], Mooren's ulcer [8] and uveitis [1,9]. However, the determination of FK506 pharmacokinetics in the eye especially in aqueous humor using LC–MS/MS is still unknown.

Several analytical methods including bioassays, enzyme immunoassays, radioreceptor monitoring, and high-performance liquid chromatographic–tandem mass spectrometry (HPLC–MS/MS) have been developed for the measurement of tacrolimus concentrations [10,11]. There are a few bioanalytical methods currently available for the quantification of tacrolimus in body fluids, and most of them are for measuring plasma concentration of tacrolimus in pharmacokinetic studies [10–15]. These methods were considered rather complicated for the analysis of samples with rather small amount of proteins such as ocular aqueous humor. Some immunoassays such as Dade Behring EMIT tacrolimus assay, the Abbott IMx tacrolimus II microparticle enzyme immune assay (MEIA) and INCSTAR Corporation Pro-Trac tacrolimus ELISA are currently available to determine tacrolimus concentrations in biological fluids such as whole blood and aqueous humor [1,15,20]. However, cross-reactivity of specific antibody to tacrolimus with its metabolites may interfere with the results in all above assays [16]. The resulted tacrolimus concentration in blood usually showed a positive deviation than that of a parent drug-specific LC–MS/MS method [14]. As for tacrolimus concentration in the eye, the previous studies were based on immunoassay for tacrolimus quantification in aqueous humor [12,20]. Until now, there is no published paper using LC–MS/MS method for the measurement of tacrolimus in the ocular aqueous humor.

The LC–MS/MS method in our study was validated to ensure accurate quantification of tacrolimus in rabbit aqueous humor above the concentration of 0.5 ng/ml. This method is sensitive,

* Corresponding author. Tel.: +86 20 87331540; fax: +86 20 87331550.
E-mail address: zhoushiy@mail.sysu.edu.cn (S.-y. Zhou).

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specific and very fast so that it can be applied to determine the very low concentration of tacrolimus in other pharmacokinetic studies.

2. Experiment

2.1. Reagents and chemicals

Tacrolimus (MW 803.5) was a gift from Fujisawa Inc. (Japan). The ritonavir used as the internal standard (IS) (MW 720.3) was obtained from Wyeth (Marietta, PA, USA). The structures of both compounds are given in Fig. 1. Methanol and acetonitrile were purchased from Tedia Company Inc. (Fairfield, OH, USA), formic acid of reagent grade from Sigma (Sigma Chemicals, St. Louis, MO, USA). Distilled de-ionized water was prepared with the Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.2. LC–MS system

The LC–MS system including the Surveyor autosampler and MS pump was purchased from Thermo Finnigan (Thermo-Finnigan, San Jose, CA, USA). A reversed-phase column, Thermo-Hypersil-BDS-C18 (3 μm, 100 mm × 2.1 mm i.d., Elite, Dalian, China) and guard column, Phenomenex ODS (4 mm × 3.0 mm i.d.) were used for all chromatographic separation at room temperature (20 °C). The mobile phase (0.1% formic acid in water, methanol and acetonitrile (5:85:10, v/v/v) was pumped at a flow rate of 0.3 ml/min.

The injection volume was 10 μl. The total run time for the analysis was 2 min. Column eluates were analyzed with a Thermo Finnigan TSQ Quantum triple quadrupole mass spectrometer (San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. The mass spectrometer was operated in a positive electrospray ionization mode. Nitrogen was used as a sheath gas and ultra-high purity helium as the dampening gas in the Q_2. The ESI source voltage was set at 3.5 kV and sheath gas flow-rate and auxiliary gas flow-rate were 35 and 5 psi, respectively; capillary temperature was 350 °C. The Q_1 and Q_3 peak widths were set to 0.7 full width at half maximum (FWHM). When running collision-induced dissociation (CID), the pressure was set to 1.5 mTorr. For selected reaction-monitoring (SRM) mode, the following transitions were recorded: FK506 m/z 826.5 $[M+Na]^+ \rightarrow m/z$ 616.2 and IS m/z $[M+Na]^+$ 743.4 $\rightarrow m/z$ 573.2, both at 35 eV. The LC system and mass spectrometer were controlled using the Thermo Finnigan Chemstation software (Xcalibur version 1.3). Data were processed using the IS method by plotting peak area ratios versus relative analyte/IS concentration with a weighting factor $1/x^2$.

2.3. Animals

Six New Zealand albino rabbits, weighing 2.2 ± 0.2 kg, were obtained from Animal Center of Zhongshan Ophthalmic Center at Sun Yat-sen University (Guangzhou, China) and were housed under standard laboratory conditions with free access to water and food. Experiments were done after the approval from the animal care committee of Sun Yat-sen University.

2.4. Sample preparation

2.4.1. Stock solution

A 400-μg/ml solution of IS was prepared in methanol and further diluted with methanol to 50 ng/ml. For calibration standards, a 400-μg/ml stock solution of FK506 was prepared in methanol and further diluted with methanol to obtain desired concentrations to spike into rabbit aqueous humor samples. The stock solutions were stored at −20 °C for future use.

2.4.2. Aqueous humor samples

Drug-free aqueous humor was obtained from the healthy rabbits and stored at −20 °C and then thawed at room temperature for use as quality control (QC) samples in making calibration curves.

Aqueous humor samples (30 μl) were spiked with 60 μl of IS stock solution (50 ng/ml). Then 60 μl of methanol was added to precipitate protein. The mixture was vortexed for 1.5 min and centrifuged at 13,000 rpm for 10 min. The formed upper organic phase (130 μl) was transferred to HPLC auto-sampler vials, and then 10 μl of the organic phase solution was injected into the LC–MS/MS system.

2.5. Calibration and linearity

60 μl of stock solution in various concentrations of tacrolimus were transferred into 1.0-ml Eppendorf tubes and evaporated to dryness under nitrogen stream. Drug-free rabbit aqueous humor

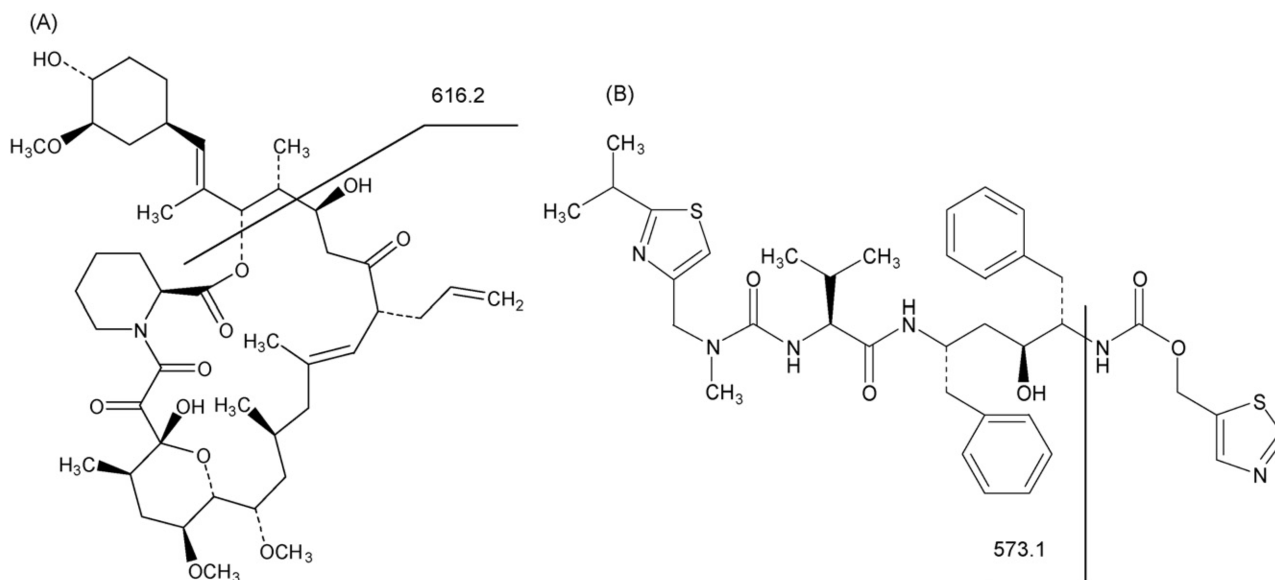

Fig. 1. Structures of tacrolimus (A) and ritonavir (B) and their possible cleavage sites.

(30 μl each) was added and mixed for 1.5 min to achieve the concentrations of 0.5, 1, 2, 10, 40, and 100 ng/ml in aqueous humor. Then each was added 60 μl methanol and 60 μl IS, vortex-mixed and centrifuged. 10 μl of the supernatant was injected. Calibration curve was triplicate. The ratio of the peak areas of FK506 to the peak areas of IS was calculated. The calibration curves were constructed by weighted ($1/x^2$) least-squares linear regression analysis of the peak area ratios of FK506/IS versus the concentrations of FK506. Calibration curve equations were used to calculate the concentrations of FK506 in the aqueous humors samples and QC samples from their peak area ratios.

2.6. Precision and accuracy

Three levels of QCs, 1.0, 10.0, and 100.0 ng/ml (low, medium, and high) as well as 0.5 ng/ml (LLOQ) were spiked into rabbit aqueous humor. These concentrations were selected based on the concentration of FK506 observed in the preliminary pharmacokinetics experiments. The intra-batch and inter-batch precision and accuracy were determined by analyzing a set of QC samples ($n = 6$) at 1.0, 10.0, and 100.0 ng/ml.

2.7. Recovery and matrix effect

Recovery and matrix effect was evaluated by analyte determination in six replicates (six different aqueous humor sources) of three different concentrations (1.0, 10.0, and 100.0 ng/ml). They were analyzed in:

- Set 1: mobile phase.
- Set 2: standard mixtures.
  Aqueous humor samples (30 μl each) from six rabbits were added 120 μl methanol each to precipitate protein, vortex-mixed and centrifuged, the upper organic phase was obtained. Then FK506 standard solutions and IS were spiked to achieve the concentration of 1.0, 10.0, 100.0 ng/ml of FK506 and 50 ng/ml of IS, respectively. The combined sample was vortex-mixed and then 10 μl of it was injected into the LC–MS/MS system.
- Set 3: aqueous humor mixtures.
  Aqueous humor samples (30 μl each) from six rabbits were spiked with FK506 to achieve the concentration of 1.0, 10.0, and 100.0 ng/ml, then each was added 60 μl methanol and 60 μl IS. The combined samples were vortex-mixed. 10 μl of the upper organic phase was injected.

All sets of procedures were conducted three times.

2.8. Stability

The stability of FK506 aqueous humor samples after undergoing three freeze–thaw cycles was evaluated. Aqueous humor samples were stored at room temperature for 4 h before processing and then stored at room temperature for 12 h after processing, respectively. The stability of aqueous humor samples and standard solutions stored at −20 °C for 15 days was also evaluated.

2.9. Pharmacokinetic studies

The LC–MS/MS method was successfully applied to pharmacokinetic studies of FK506 in aqueous humor of rabbits. Aqueous humor was collected from the rabbits before and after receiving a single dose of FK506 in eye drops. Approximately 50 μl of aqueous humor was collected in Eppendorf tubes before drug administration and post-treatment at 30, 60, 90, 105, 120, 150, 180, 210, 270, 360, and 480 min, respectively. Six experiments using six rabbits were done for each compound in the pharmacokinetic study. All aqueous humor samples were sealed and stored at −20 °C until analysis. The aqueous humor samples were extracted as above mentioned.

3. Results and discussion

3.1. Sample preparation

Sample preparation is a key procedure for determination of analytes in biological samples. After several trials, a protein-precipitation using neat methanol was found to be appropriate for the determination of FK506 in rabbit aqueous humor. Rather than other solvent, methanol was selected to be a protein-precipitation agent for compatibility with the mobile phase to produce symmetric peak shapes for the analytes and IS. Compared to previous methods [11,18,19], our methodology used a rather simple sample preparation procedure to eliminate possible disturbing substances for FK506 LC–MS/MS determination. A clean chromatogram was

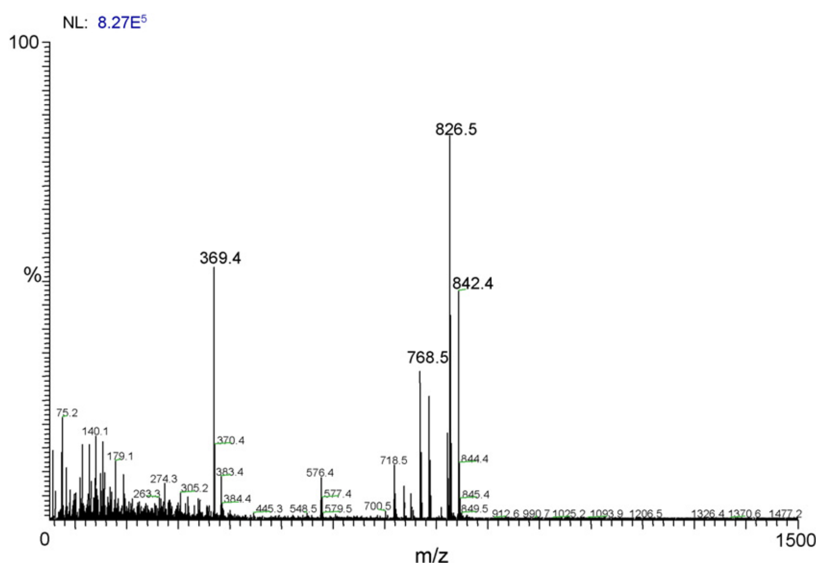

Fig. 2. Full scan spectra of FK506 after positive ionization from infusion.

achieved because there are much smaller amount of proteins in aqueous humor compared to other samples (such as plasma samples), accordingly, a simpler sample preparation procedure was conducted and satisfied results were obtained.

3.2. Determination of HPLC–MS/MS settings

HPLC–MS/MS is a powerful technique and now is widely used in biological analysis. A Thermo-Hypersil-BDS-C18 column was selected for the chromatographic separation because it exhibited excellent peak shape and had sufficient response for FK506.

Other chromatographic conditions, especially the composition of ionization agent in mobile phase, were optimized through several trials to obtain good intensity of the response and symmetric peaks. Formic acid, acetic acid, and ammonium acetate were tested as ionization agent candidates, and it was found that formic acid performed better than the other two in terms of intensity of the response. Changing the percentage of the ionization agent buffer in the mobile phase affected peak symmetry and the resolution between the analyte peak and the dead volume peak. Buffers with the percentage in the mobile phase in the range 2–10% were tested, and 5% was found to be optimal. The final composition of the mobile phase was 0.1% formic acid in water, methanol and acetonitrile (5:85:10, v/v/v). An internal standard was necessary for the determination of analytes in biological samples and ritonavir was found to be optimal for an IS because of its stability in aqueous humor and similarity of chromatographic behavior with FK506. The capacity factors and asymmetry factors for FK506 are 1.88 and 1.44, for IS are 1.64 and 1.86, respectively. No late eluting peaks that would cause interference with subsequent injections was detected.

Signal intensity for both FK506 and IS in rabbit aqueous humor was found to be optimal in a positive mode of ESI. Fig. 2 shows the full scan spectra of FK506 after positive ionization. An ion of m/z = 826.5 that corresponds to a capture of a sodium ion ($[M+Na]^+$) of FK506 was more intensive than that of $[M+H]^+$, $[M+K]^+$ ones. Fig. 3 shows the spectra of $[M+Na]^+$ MS/MS of FK506 and IS. The sodium adducts of FK506 and IS showed good stability throughout the whole process of method validation which was similar to the results of other investigators [18,19]. The chemical structures of tacrolimus and IS as well as their possible cleavage sites were shown in Fig. 1.

3.3. Method specificity and selectivity

Method specificity towards endogenous rabbit aqueous humor matrix blank aqueous 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 IS solution. Chromatograms of blank aqueous humor and aqueous humor samples spiked with FK506 (0.5 and 10 ng/ml) and IS (100.0 ng/ml each) were compared to show the specificity and selectivity of the proposed procedure. Method selectivity and acceptability of

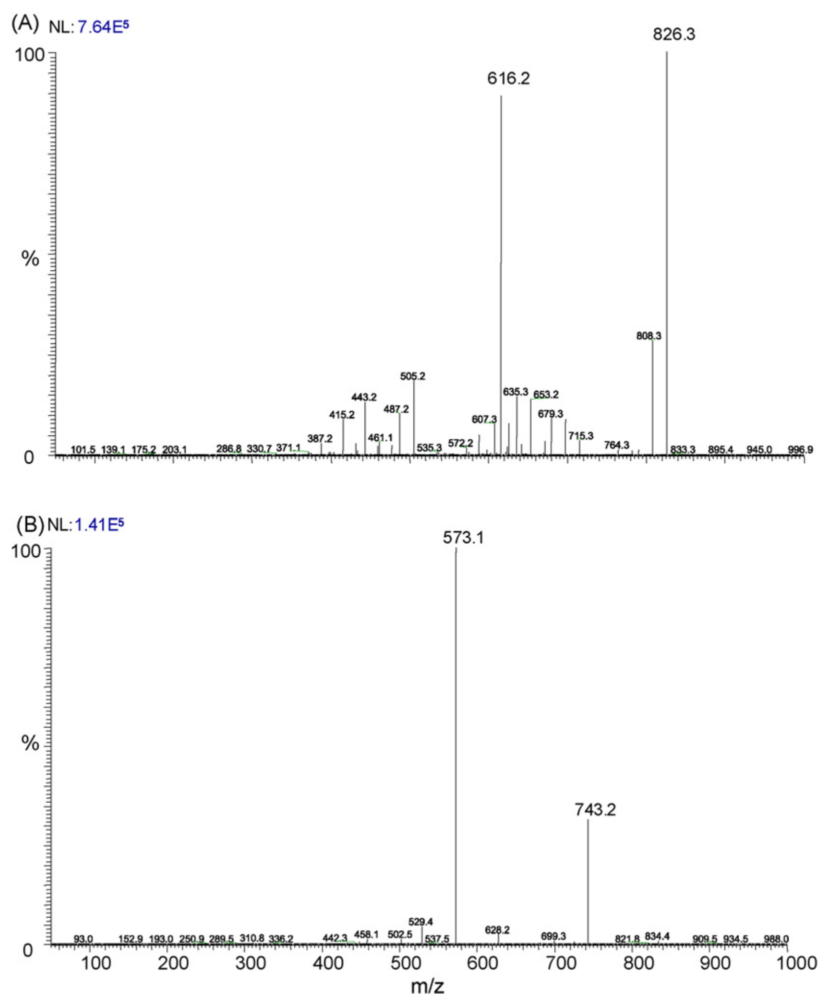

Fig. 3. Product ion mass spectra of [M+Na] + ions of tacrolimus (A) and ritonavir (B) from LC–MS/MS.

blank matrix samples were evaluated based on a determination of whether a significant interference was found in each analyte's SRM trace. Selectivity and blank matrix samples were considered to be acceptable if no discernible peak at the retention time of FK506 was observed or if the height and area response of a discernible peak was less than 20% of the height and area response of the closest, in run sequence, LLOQ standard [17]. In Fig. 4, the retention times of FK506 and IS were 1.4 and 1.3 min, respectively, and no interferences were observed at the SRM mode for the analytes tested.

In addition, the "cross-talk" between MS/MS channels used for monitoring FK506 and IS was assessed by the following procedure: separately injecting FK506 (10.0 ng/ml) and monitoring the response in the IS channel and by injecting an aqueous humor sample spiked only with IS and monitoring the response in the FK506 channel. No "cross-talk" between channels was observed which suggested that no interferences were present from the IS that could contribute to the analyte m/z channel, and vice versa.

3.4. Linearity, accuracy, and precision

The six-point calibration curve obtained by weighted linear regression ($1/x^2$) showed good linearity over the whole concentration range (0.5–100.0 ng/ml), which covered the concentrations typically found in plasma after administration of FK506 in the pharmacokinetic study. The correlation coefficient was better than 0.99 ($n = 6$). Table 1 gives a summary of the response linearity.

Table 1
Summary of linearity

Run number	Equation form: $Y = BX + A$		Correlation coefficient (r)
	B	A	
1	0.04632	0.00720	0.99669
2	0.05134	0.00835	0.99725
3	0.05219	0.00736	0.99740
Mean	0.04995	0.00763	0.99711
S.D.	0.00317	0.00062	0.00037

The lower limit of quantification was defined as the lowest concentration on the calibration curve in which an acceptable accuracy of ±20% and a precision below 10% were obtained. The present LC–MS/MS method offered a LLOQ of 0.5 ng/ml in rabbit aqueous humor sample. Under the present LLOQ of 0.5 ng/ml, the concentration of FK506 can be determined in aqueous humor samples after a single dose of 25 μg FK506 eye drops which is sensitive enough for the pharmacokinetic study of FK506 in the eye.

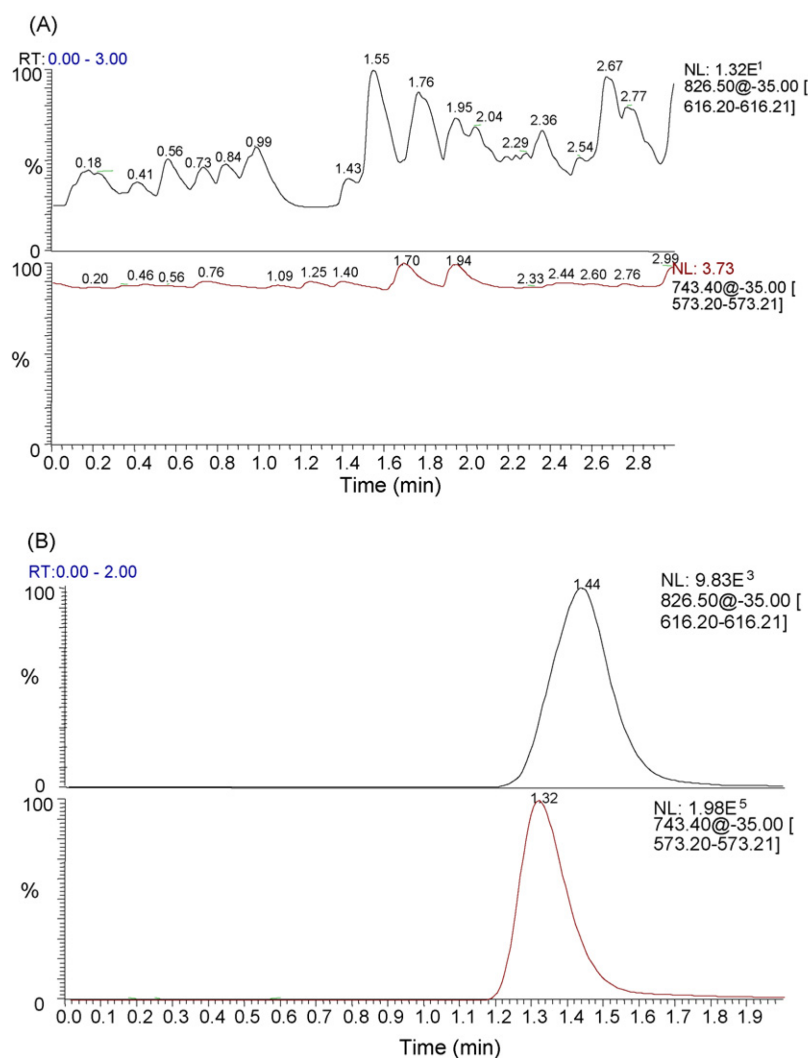

Fig. 4. Representative SRM chromatograms of FK506 (upper) and IS (lower) in aqueous humor. (A) Blank aqueous humor sample; (B) aqueous humor sample spiked with FK506 (0.5 ng/ml, LLOQ) and IS (100.0 ng/ml); (C) aqueous humor sample spiked with FK506 (10.0 ng/ml, QC2) and IS (100.0 ng/ml); (D) aqueous humor sample obtained at 120 min after administration from no. 1 rabbit.

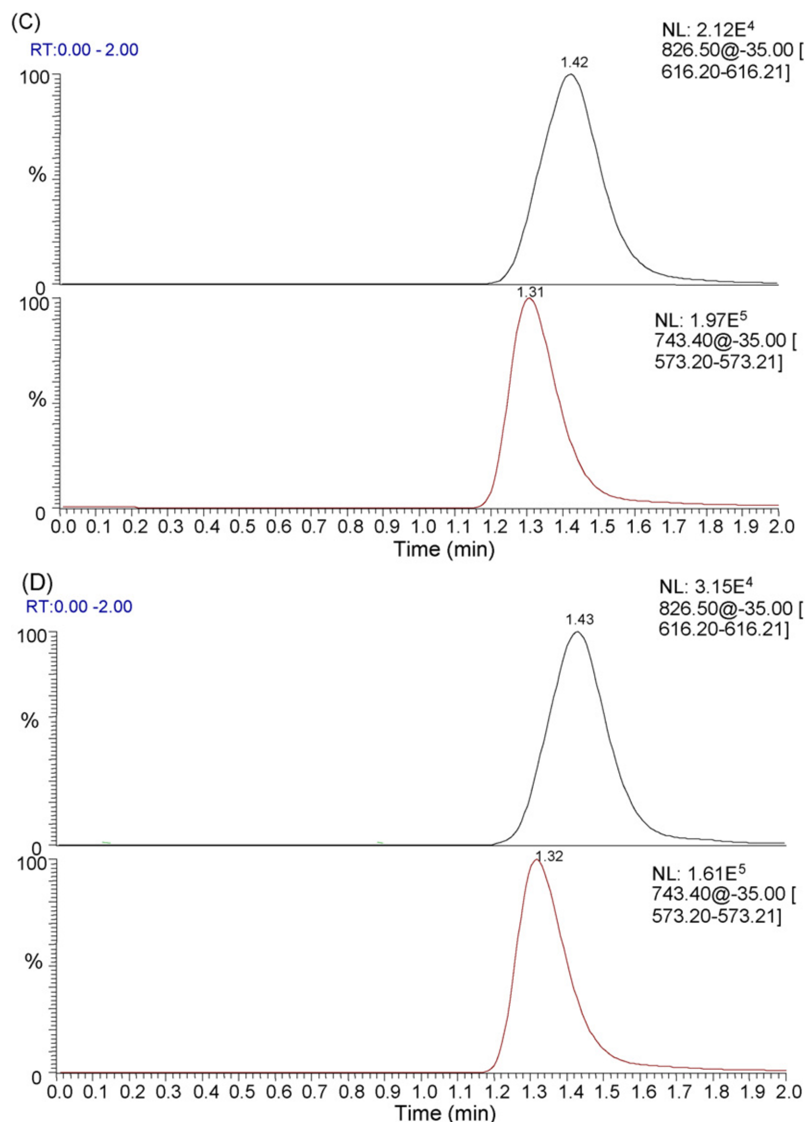

Fig. 4. (*Continued*).

Inter-run and intra-run assay were performed to evaluate precision (R.S.D.) and accuracy (% deviation). Within-run precision and accuracy was determined by analyzing six series of four aqueous humor samples (S6, S4, and S2). Intra-batch precision and accuracy were assessed by the analysis of three aqueous humor samples (S6, S4, and S2) in six series. The results are summarized in Table 2 and prove acceptable precision and accuracy of the proposed method.

Table 2
Summary of accuracy and precision of LLOQ and QC samples

Amount added (ng/ml)	0.50	1.00	10.0	100.0
Batch	1	3	3	3
n per batch	6	6	6	6
n total	6	18	18	18
Mean (ng/ml)	0.51	0.97	9.9	101.0
S.D.	0.04	0.10	0.5	3.9
% deviation[a]	7.95	−2.51	−0.65	1.03
Inter-run R.S.D. (%)[b]	1.80	10.52	5.79	3.16
Intra-run R.S.D. (%)[b]	1.80	1.78	3.03	7.07

[a] Accuracy.
[b] Precision.

3.5. Recovery and matrix effect

In LC–MS analysis, the recovery can be influenced by two different effects. Firstly, the process during sample preparation. Secondly, possible matrix effects, as ion suppression or ion enhancement, particularly when working with analytes present at ng/ml levels in biological samples and in the view of very simple sample pre-treatment method as well as very short run. Results of evaluation on extraction recovery and matrix effect are listed below.

3.5.1. Recovery

Three concentration levels were tested as specified for the concentration of 1.0, 10.0, and 100.0 ng/ml. Recovery values for each analyte and IS were determined by calculating the ratios of the raw peak areas of the pre-extraction spiked samples (set 3) to that of the samples spiked after extraction (set 2). The results are indicated in Table 3. The data suggested that the extraction efficiency and uniformity met the parameters required for a valid bioanalytical method.

Table 3
Summary of recovery from QC samples and internal standard

	FK506 concentration (ng/ml)			IS cone (ng/ml)
	1.00	10.0	100.0	100.0
Percentile	102.4	104.3	101.7	97.5
	108.6	103.5	100.1	100.5
	106.3	106.2	100.1	100.3
Mean (%)	105.8	104.7	100.6	99.4
S.D.	1.9	1.4	0.9	1.7

3.5.2. Matrix effect

The susceptibility of our methodology to ion suppression/enhancement was evaluated using the procedure described in Section 2.7. The results of were summarized in Table 4.

The absolute matrix effect (ME), i.e. the possibility of ionization suppression or enhancement and overall process efficiency (PE) were evaluated by comparing the results of analysis of three sets of samples as follows [21]:

$$\text{ME}\ (\%) = \frac{(2)}{(1)} \times 100, \quad \text{PE}\ (\%) = \frac{(3)}{(1)} \times 100.$$

where (1) is the mean peak area for concentration of set 1, (2) is the mean peak area for concentration of set 2, and (3) is the mean peak area for concentration of set 3.

As can be seen in Table 4, ion suppression/enhancement is not a serious issue with our methodology (with an average of 4% ion suppression), at least for the six aqueous humor samples examined in this work. An overall process efficiency of 97.2% with low variability were found to be consistent over the calibration range, consequently the published method was proved to be reliable.

3.6. Stability

Stability assessments under different conditions [freeze–thaw (FT) and short-term (ST) bench top room temperature as well as the long-term (LT) storage stability] were established. The results indicated that each analyte had an acceptable stability under those conditions, as shown in Table 5.

3.7. Pharmacokinetics of FK506

The LC–MS/MS method showed satisfactory determination of FK506 in rabbit aqueous humor and can be successfully used for the pharmacokinetic study of tacrolimus eye drop in rabbits. The concentration–time profiles for FK506 are shown in Fig. 5.

Table 4
Summary of absolute matrix effect, relative matrix and process efficiency

	FK506 concentration (ng/ml)		
	1.00	10.0	100.0
Set 1			
Mean peak area	134,219	268,956	1,654,368
S.D. (%)	1.7	1.2	0.8
Set 2			
Mean peak area	129,569	256,987	1,608,543
S.D. (%)	1.9	1.5	0.9
Set 3			
Mean peak area	130,947	260,349	1,609,955
S.D. (%)	1.2	1.0	0.7
ME (%)	96.5	95.5	97.2
PE (%)	97.6	96.8	97.3

Table 5
Summary of freeze–thaw (FT), short-term (ST) bench top room temperature and the long-term (LT) storage stability

Item	Nominal amount (ng/ml)	Mean amount found[a] (ng/ml)	Relative deviation (%)
FT	1.00	1.04	4.2
	10.0	9.2	−8.2
	100.0	95.7	−4.3
ST			
4 h[b]	1.00	0.95	−4.9
	10.0	10.3	2.5
	100.0	106.4	6.4
12 h[c]	1.00	0.96	−3.6
	10.0	10.7	6.6
	100.0	103.0	3.0
LT			
Sample[d]	1.00	1.00	0.4
	10.0	9.9	−1.3
	100.0	99.9	−0.1
	0.50	0.43	14.4
Stock solution[e]	5.0	4.4	12.9
	50.0	51.5	−2.9

[a] Each concentration tested three times.
[b] Samples stored at room temperature for 4 h.
[c] Post-processed stored at auto-sampler for 12 h.
[d] Samples stored at −20 °C for 15 days.
[e] Stock solution stored at −20 °C for 15 days.

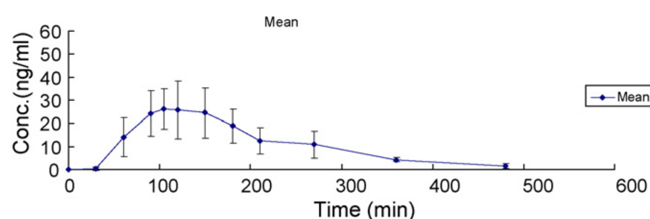

Fig. 5. Pharmacokinetic profile of tacrolimus following a single dose (25 μg) eye drops to rabbits.

3.8. Comparison with former study

In the previous study of our group [20], the pharmacokinetics of FK 506 in nanoparticles in rabbit aqueous humor have been determined using the Pro-Trac tacrolimus ELISA kit. The limit of detection (LOD) with that study was 0.3 ng/ml, however, LLOQ has not been evaluated. In terms of precision, a positive bias of 15% at the concentration of 1.0 ng/ml and 11% at 10.0 ng/ml compared to that of LC–MS/MS were evident which was similar to that reported by some investigators [15,22].

4. Conclusion

A method was developed for quantification of tacrolimus in rabbit aqueous humor by HPLC–MS/MS. This method with LLOQ of 0.5 ng/ml was fast and just took 2 min. There were no interferences found from endogenous aqueous humor components or other sources and no "cross-talk" observed in aqueous humor samples. This assay has showed consistent precision and accuracy. The fact that no gradient elutions and no chromatographic separations are used eliminated the need of "fine tuning" of the HPLC part of the method, which makes this method suitable for easy transfer and implementation between different laboratories. The analytical method presented here has been proved useful and more reliable compared to the ELISA for the determination of FK506 concentration in ocular aqueous humor in pharmacokinetic studies.

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