Journal of Chromatography B, 867 (2008) 111–118

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Rapid and simultaneous determination of tacrolimus (FK506) and diltiazem in human whole blood by liquid chromatography–tandem mass spectrometry: Application to a clinical drug–drug interaction study

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ARTICLE INFO

Article history:
Received 2 February 2008
Accepted 26 March 2008
Available online 1 April 2008

Keywords:
Tacrolimus
Diltiazem
Liquid chromatography–tandem mass spectrometry
Pharmacokinetics
Renal transplant

ABSTRACT

Tacrolimus (FK506) is a potent immunosuppressant widely used for organ transplantation patients while diltiazem (DTZ), a calcium-channel inhibitor, is often used in renal transplantation patients to prevent post-transplant hypertension. However, DTZ has a significant pharmacokinetic interaction with FK506. In this study, a rapid and sensitive ammonium-adduct based liquid chromatography–tandem mass spectrometry (LC/MS/MS) method has been developed and validated for the simultaneous determination of FK506 and DTZ in human whole blood using ascomycin as the internal standard (IS). After extraction of the whole blood samples by ethyl acetate, FK506, DTZ and the IS were subjected to LC/MS/MS analysis using electro-spray positive-ion mode ionization (ESI^+). Chromatographic separation was performed on a Hypersil BDS C18 column (50 mm × 2.1 mm, i.d., 3 μm). The MS/MS detection was conducted by monitoring the fragmentation of 821.7 → 768.9 (m/z) for FK506, 415.5 → 310.3 (m/z) for DTZ and 809.8 → 757.0 (m/z) for IS. The method had a chromatographic running time of approximately 2 min and linear calibration curves over the concentrations of 0.5–200 ng/mL for FK506 and 2–250 ng/mL for DTZ. The recoveries of liquid–liquid extraction method were 58.3–62.6% for FK506 and 50.4–58.8% for DTZ. The lower limit of quantification (LLOQ) of the analytical method was 0.5 ng/mL for FK506 and 2 ng/mL for DTZ. The intra- and inter-day precision was less than 15% for all quality control samples at concentrations of 2, 10, and 50 ng/mL for FK506 and 5, 25, and 100 ng/mL for DTZ. The validated LC/MS/MS method has been successfully used to analyze the concentrations of FK506 and DTZ in whole blood samples from pharmacokinetic studies in renal transplanted patients.

1. Introduction

The success of organ transplantation is highly dependent on the dose of immunosuppressant drugs administered to the patient. In turn, proper dosing requires rapid and reliable quantification of the immunosuppressants present in the patient's whole blood. The primary reasons for this are a narrow therapeutic range, variations in intra- and inter-individual pharmacokinetics and the lack of a reliable correlation between dose and drug exposure [1–3]. Consequently, the dose of immunosuppressant needs to be tailored to the individual patient, necessitating therapeutic drug monitoring.

Tacrolimus (FK506) is a well-known potent immunosuppressant that has been widely used for solid organ transplantation patients because of its perceived advantages over cyclosporine for nearly two decades [4–7]. As with cyclosporine, however, tacrolimus is potentially nephrotoxic, limiting its usefulness in kidney transplantation. Based on the intracellular mechanisms of tacrolimus, calcium-dependent mechanisms may be important during the development of tacrolimus-related nephrotoxicity. Previous studies demonstrated that the calcium-channel inhibitor diltiazem (DTZ) brings about not only functional, but also morphological improvement in tacrolimus-induced acute nephrotoxicity, and effective in the prevention of ischemic damage [8–10]. However, diltiazem has a significant pharmacokinetic interaction with tacrolimus [8,11]. It is known that the metabolism of tacrolimus

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doi:10.1016/j.jchromb.2008.03.024

occurs in the liver [12] and the small intestine [13] via the cytochrome P450 (CYP3A4). Therefore, inhibition or induction of CYP3A4 by coadministered drugs will lead to clinically significant pharmacokinetic drug interactions [14]. This effect is reinforced by the narrow therapeutic index and by inter- and intra-individual variations in pharmacokinetics. DTZ is also a CYP3A4 substrate and a potent CYP3A4 and P-glycoprotein inhibitor [11]. It has been shown in humans that plasma concentrations of DTZ increased after administration of multiple doses [15]. The reason could be attributed to inhibition of hepatic and intestinal CYPs (e.g. CYP3A4 and CYP2D6) and intestinal P-glycoprotein by DTZ and/or its metabolites [16–21].

Therefore, there has been an increased demand for therapeutic monitoring of tacrolimus [22] and DTZ coupled with a requirement for increased analytical sensitivity. To date, numerous methods for the separate determination of tacrolimus or DTZ in biological samples have been reported, but there is no one method for simultaneous detection of these two drugs. As such, we developed a rapid and sensitive LC/MS/MS method to simultaneously determine tacrolimus and diltiazem in human whole blood using a simple procedure of protein precipitation followed by liquid–liquid extraction. In this study, we chose electro-spray ionization (ESI^+) as the ionization source. Our method exhibited excellent performance in terms of high selectivity, robustness and excellent efficiency (2 min per sample) with simplicity of sample preparation. This LC/MS/MS method was successfully applied to a pharmacokinetic interaction study of tacrolimus and DTZ in renal transplanted patients.

2. Experimental

2.1. Chemicals and reagents

Tacrolimus (FK506, Fig. 1) with a purity of 98% as determined by HPLC with ultraviolet (UV) detection was synthesized and provided by Toronto Research Chemicals Inc. (Toronto, Canada). Ascomycin (FK520, IS, Fig. 1) with a purity of 95% as determined by HPLC with UV detection was synthesized and provided by BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA). Ascomycin is a structural analog of FK506 with similar molecular weight and hydrophobicity and thus it was used as an internal standard. The chemical structures were verified by ^1H nuclear magnetic resonance analysis and compared with reference compounds. Diltiazem hydrochloride (Fig. 1) with a purity of 100.0% was obtained from Guangzhou Institute of Pharmaceutical Industry (Guangzhou, China). Methanol and acetonitrile of HPLC grade were purchased from Tedia Inc. (Beijing, China). All other reagents were of analytical grade or HPLC grade when appropriate. Human whole blood treated with the anticoagulant sodium citrate from healthy blood donors was obtained from the Central Laboratory of the Blood Transfusion Service of Guangzhou, Guangdong, China. The ethics approval was obtained from the Human Investigation Ethics Committee of SunYat-sen University, Guangzhou, China. Ultra-pure water was obtained from a Milli Q-plus system (Billerica, MA).

2.2. Preparation of standard and quality control samples

The stock standard solutions of FK506 and DTZ were prepared by dissolving accurately weighed individual compounds in methanol–water (50:50, v/v) to give a final concentration of 500 μg/mL and 2 mg/mL, respectively. The solutions were then serially diluted with methanol–water (50:50, v/v) to obtain working solutions at concentrations over 0.025–10.0 μg/mL for FK506 and 0.032–4.2 μg/mL for DTZ. A standard stock solution of FK520 (IS) at 100 μg/mL was also prepared in methanol–water solution (50:50, v/v) and then diluted to obtain a working solution at 1 μg/mL. The stock solutions of each analyte or IS were stored at −80 °C, and

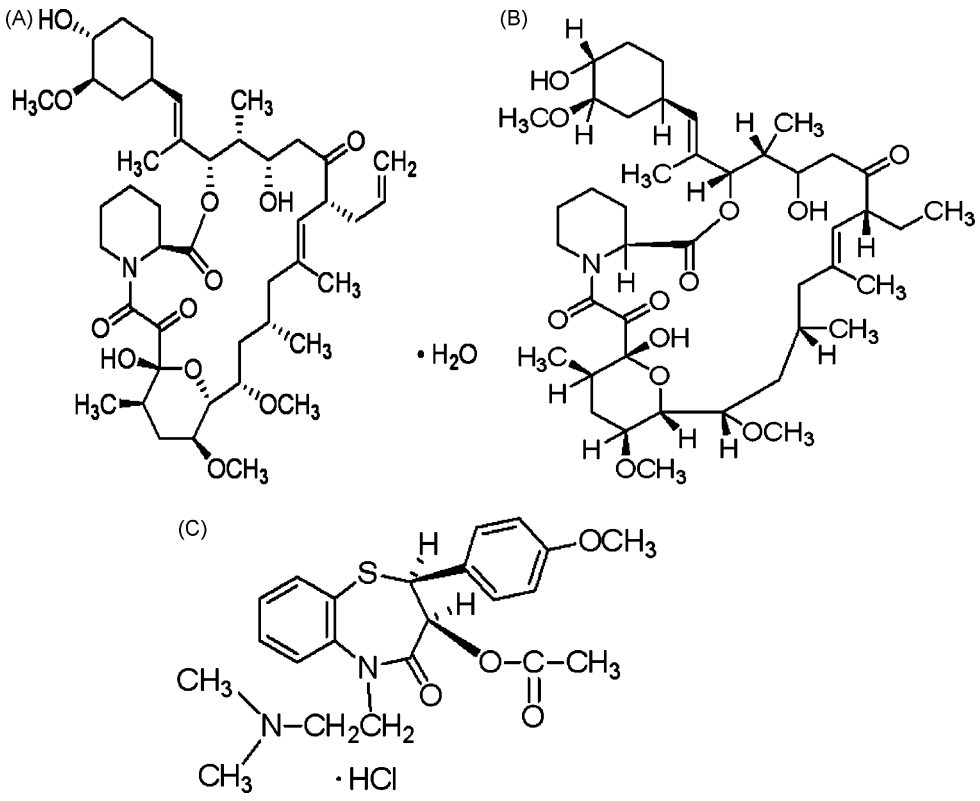

Fig. 1. Chemical structures of tacrolimus (A), ascomycin (B), and diltiazem hydrochloride(C).

the working solutions were stored at 4 °C and were brought to room temperature before use. The analytical standard and quality control (QC) samples were prepared by spiking blank human whole blood with standard working solutions during validation and each experimental run for the pharmacokinetic study. Calibration samples were made at concentrations of 0.5, 2, 5, 10, 25, 50, 100, 150 and 200 ng/mL for FK506 and 2, 5, 10, 25, 50, 100, 150, 200 and 250 ng/mL for DTZ. Quality control samples were prepared at concentrations of 2, 10, and 50 ng/mL for FK506 and 5, 25, and 100 ng/mL for DTZ.

2.3. Sample preparation

Standard working solutions (10 μL FK506, 30 μL DTZ and 10 μL IS) were added into 0.45 mL blank human whole blood sample in 10 mL centrifuge tubes, and the whole blood samples were vortexing-mixed. Zinc sulfate solution (0.1 M, 1 mL) was added and mixed well to lyse the cells, this was followed by adding 1 mL acetonitrile and vortexing-mixed for 1 min and standing at room temperature for 10 min. After centrifugation at 2500 *g* for 10 min, the supernatant was transferred to a clean centrifuge tube and extracted with 3 mL extraction solvent ethyl acetate. After vortex-mixing for 1 min and standing at room temperature for 10 min, the mixtures were centrifuged at 2500 *g* for 10 min. The organic phase was then transferred to a clean centrifuge tube and evaporated to dryness. The residues were dissolved in 200 μL mobile phase and an aliquot (10 μL) of the reconstituent was injected onto the LC/MS/MS for analysis.

2.4. Liquid chromatographic and mass spectrometric conditions

A Waters 2695 separation module (alliance) (Avondale, CA) was used for solvent and sample delivery. A chromatographic separation was achieved by using a C18 column (Hypersil BDS C18, I.D. 2.1 mm × 50 mm, 3 μm, Elite HPLC Inc., Dalian, China) at room temperature. The mobile phase consisted of methanol–water (containing 2 mM ammonium acetate) (95:5, v/v), pumped at a flow rate of 200 μL/min. The total running time was 2 min for each sample. A Quattro microTM triple quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an ESI source was used for mass analysis and detection. Mass spectrometric analysis was performed in the positive-ion mode (ESI^+) and set up in the multiple reaction monitoring (MRM) mode. Nitrogen was used as desolvation (550 L/h) and nebuliser (50 L/h) gas. Argon was used as collision gas (0.0033 mbar). The capillary voltage was 3.0 kV for the analytes and the entrance and exit energies of the collision cell were set at +1 and +2, respectively. The source and desolvation temperatures were kept at 110 and 350 °C, respectively. On the basis of the full-scan mass spectra of each analyte, the most abundant ions were selected and the mass spectrometer was set to monitor the transitions of the precursors to the product ions as follows: m/z 821.7 → 768.9 for FK506, m/z 415.5 → 310.3 for DTZ and m/z 809.8 → 757.0 for IS. The dwell time per channel was set to 0.8 s for FK506 and IS and 0.3 s for DTZ. The optimized ESI^+-MS/MS parameters are listed in Table 1. The system was controlled by Masslynx V 4.0 software (Micromass, Manchester, UK).

2.5. Method validation

The method was validated for selectivity, accuracy, precision, recovery, calibration curve and reproducibility according to the FDA guideline for validation of bioanalytical methods [23]. The selectivity was investigated by preparing and analyzing six individual human whole blood samples at the LLOQ. The LLOQ was defined as the lowest concentration on the calibration curve of the analytes measured with acceptable precision and accuracy (i.e. relative standard deviation (RSD, refers to the absolute value of the coefficient of variation (CV)) and relative error < 20%) and with at least five times the response compared to blank response (noise). The limit of detection (LOD) was estimated as the amount of FK506 or DTZ which caused a signal three times to noise. Linearity was assessed by preparing and analyzing FK506 and DTZ standard samples over 0.5–200 and 2–250 ng/mL, respectively, with nine concentration points in human whole blood.

Calibration curves were generated using the analyte to IS peak-area ratios by weighted $1/X$ and $1/X^2$ for FK506 and DTZ, respectively, on five consecutive days. The acceptance criteria for a calibration curve were, a correlation coefficient (r) of 0.99 or better, and that each back-calculated standard concentration must be within15% deviation from the nominal value except at the LLOQ, for which maximum acceptable deviation was set at 20%. Accuracy and precision were assessed by determining QC samples at three concentration levels (five samples for each concentration) on three different validation days. The precision was determined as the RSD% or CV% and the accuracy was expressed as a percentage of the measured concentration over the nominal (theoretical) concentration. The criteria used to assess the suitability of precision and accuracy was as follows: the RSD did not exceed 15% and the accuracy was within 15% of the actual value. The recovery (extraction efficiency) of analytes from human whole blood after the extraction procedure was determined by comparing the peak areas of extracted analytes with those of blank whole blood extracts spiked post-extraction. During routine analysis, each analytical run included a blank plasma, a blank plasma spiked with IS, a set of calibration samples, a set of QC samples and unknowns. The stability of analytes was assessed by determining QC samples at three concentrations with five samples for each concentration, exposed to different time and temperature conditions. The stability studies included: (a) stability at room temperature for 4 h; (b) stability after three freeze-thaw cycles; (c) stability of the extracted samples at room temperature for 24 h; and (d) the long-term stability after storage at −20 °C for 1 month.

The absolute and relative matrix effects (MEs) on the spectral response of FK506, DTZ and IS were assessed as described by Matuszewski et al. [24] with slight modifications as mentioned previously [25]. Since ME is a concern with the fast isocratic system, the co-elution effect and potential ion suppression were evaluated. To assess the absolute matrix effect, six different batches of blank whole blood was spiked with each analyte or IS. QC samples at three concentration levels (five samples for each concentration) and IS at working concentration (20 ng/mL) were analysed. The corresponding peak areas of the analyte or IS in spiked whole blood post-extraction (A) were then compared to those of the aqueous

Table 1
Selected ion transitions (m/z values) and optimized mass spectrometric parameters for the LC/MS/MS analysis of tacrolimus (FK506), diltiazem (DTZ) and ascomycin (FK520, IS) in multiple reaction monitoring (MRM) mode (positive ionization)

Analyte	Precursor ion (m/z)	Product ion (m/z)	ESI capillary voltage (kV)	Cone voltage (V)	Collision energy (eV)
FK506	821.7	768.9	3.0	20	20
DTZ	415.5	310.3	3.0	20	30
IS	809.8	757.0	3.0	20	20

standards in mobile phase (B) at equivalent concentrations. The ratio ($A/B \times 100$) is defined as the ME. An ME value of 100% indicates that the response in the mobile phase and in the whole blood extracts was the same and no absolute matrix effect was observed. A value of >100% indicates ionization enhancement, and a value of <100% indicates ionization suppression. The assessment of the relative ME was made by a direct comparison of the analyte or IS peak-area values between different lots (sources) of whole blood. The variability in the values, expressed as RSD (%), is a measure of the relative ME for the target analyte or IS.

2.6. Assay application to pharmacokinetic studies

The present method was used to determine the concentration-time profiles of FK506 (capsule, Fujisawa Ireland Limited, Killorglin, Ireland) in renal transplantation patients after oral administration of DTZ (tablet, Neptunus Pharmaceutical Co., Ltd., Shenzhen, China). Briefly, six patients (four males, two females) were involved in the study. The average age was 37.4 ± 17.3 years (range: 28–57) and the average body weight was 61.8 ± 19.3 kg. Informed consent was obtained from all subjects after explaining the aims and risks of the study. The study protocol was approved by the Human Investigation Ethics Committee of Sun Yat-sen University, Guangzhou, China. After oral administration of FK506 alone for two weeks followed by collecting the whole blood samples, they took FK506 and DTZ together for two weeks followed by collecting the whole blood samples. FK506 (0.065 mg/kg) was administrated twice a day, and 30 mg DTZ was taken three times a day. Venous whole blood samples (2 mL) were collected into EDTA-containing tubes according to the following schedule: immediately before drug administration and 0.5, 1, 2, 3, 4, 6, 9, and 12 h after dosing. The whole blood samples were clearly labeled and kept frozen at −20 °C until analysis. The whole blood concentration-time profiles of FK506 and DTZ were obtained for each individual subject, and noncompartmental pharmacokinetic parameter calculations were performed using the NONMEM Program version 1.1 (Globo-Max Inc., Ellicott City, MD). The time to peak whole blood concentration (T_{max}) and peak whole blood concentration (C_{max}) and the whole blood concentration before FK506 and DTZ were administrated (C_0) were read directly from the observed concentration versus time profiles. The area under the plasma concentration-time curve to the last measurable concentration ($AUC_{0-12\,h}$) was calculated using the linear trapezoidal rule for the observed values from zero time to the last measured time point.

2.7. Statistical analysis

Statistical analysis was performed in SPSS system for Windows version11.0. Data were expressed as mean ± SD. The comparison of pharmacokinetic parameters in six individuals with or without coadministration of DTZ was conducted using a Wilcoxon Signed-Rank Test. $P < 0.05$ was regarded as being of statistical significance.

3. Results and discussion

FK506 is present in low levels in biological fluids (therapeutic range 5–10 ng/mL for 12-h trough whole blood concentrations was controlled in this study). Such narrow therapeutic window makes frequent therapeutic drug monitoring necessary, in order to adjust the dose to prevent rejection and toxicity. Immunoassay methods are most widely employed as routine therapeutic drug monitoring of FK506 for patients. However, immunoassay methodology has relatively low sensitivity and often lacks specificity, thus is unsuitable for pharmacokinetic studies. Immunoassay also has difficulty differentiating active metabolites from its parent drug [26]. Unfortunately, the molecule has no chromophores and no intrinsic fluorescence and does not easily volatilize [27]. Therefore, HPLC with UV or fluorescence detection, which are often used as the "gold standard" in therapeutic drug monitoring, become inadequate for FK506 measurement. On the other hand, the calcium-channel blockers DTZ is widely used in the treatment of post-transplant hypertension. However, DTZ causes significant pharmacokinetic interaction with tacrolimus [8,11,29]. In the study by Jones et al. [28], whole blood FK506 concentrations were measured by microparticulate enzyme immunoassay, and the plasma concentrations of DTZ were measured by HPLC method. Thus, a rapid and sensitive LC/MS/MS method to simultaneously determine tacrolimus and DTZ in human whole blood was developed in order to simplify the process of sample preparation and detection as well as save whole blood.

3.1. Method development

In this study, the positive-ion mode (ESI^+) was chosen as the ionization source. Consistent with other studies, due to relatively weak proton affinity, FK506 and IS had low abundance of protonated molecular ions that were most often used for mass spectrometric detection in positive ion-spray mode [26]. However, the two compounds demonstrated a strong ability to bind with sodium ion (Na^+) and potassium ion (K^+) to form sodium or potassium-adduct ions. Singly charged sodium-adduct ions or potassium-adduct ions had peak (m/z) at ($M_r + 23$) or ($M_r + 39$). Although these two adducts were easily formed in the ion source, they were not suitable for MS/MS detection because of their poor fragmentation. Therefore, ammonium acetate was selected to add in the mobile phase to form ammonium-adduct ions, which had a peak (m/z) at ($M_r + 18$) and easily fragmented during MS/MS detection. Different concentrations of ammonium acetate alone or in combination with 0.1% formic acid were added in the mobile phase. It was found that the sensitivity of ammonium-adduct ions were low when formic acid was added, and the maximum sensitivity was achieved when 2 mM ammonium acetate was added. DTZ was easy to form protonated molecular ions even in the presence of ammonium acetate. Fig. 2 presents the MS/MS spectra of FK506, DTZ and IS. Therefore, the multiple reaction monitoring (MRM) transition of m/z 821.7 → 768.9 for FK506, m/z 415.5 → 310.3 for DTZ and m/z 809.8 → 757.0 for IS were selected to obtain maximum sensitivity. The proportion of organic solvent and water in the mobile phase were optimized through several trials to achieve good resolution and symmetric peak shapes, as well as shorter running time for the analyte and the IS. A mobile phase consisting of methanol–water (containing 2 mM ammonium acetate) and the ratio of 95:5 (v/v) could achieve this purpose and was finally adopted as the mobile phase.

Sample preparation is a critical step for accurate and reliable LC/MS/MS assays, but there is no one method for simultaneous extracting FK506 and DTZ from whole blood. The low polar character of the two compounds makes it possible to extract from whole blood by conventional liquid–liquid extraction technique. The extraction efficiency of different solvents including ethyl acetate, dichloromethane alone or in combination with 1% isoamyl alcohol was compared during our method development. Ethyl acetate gave the highest extraction recovery and generated the least decrease in spectral response of FK506 and DTZ and thus it was chosen as the extraction solvent in the study.

3.2. Method validation

3.2.1. Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank human whole blood with the spiked

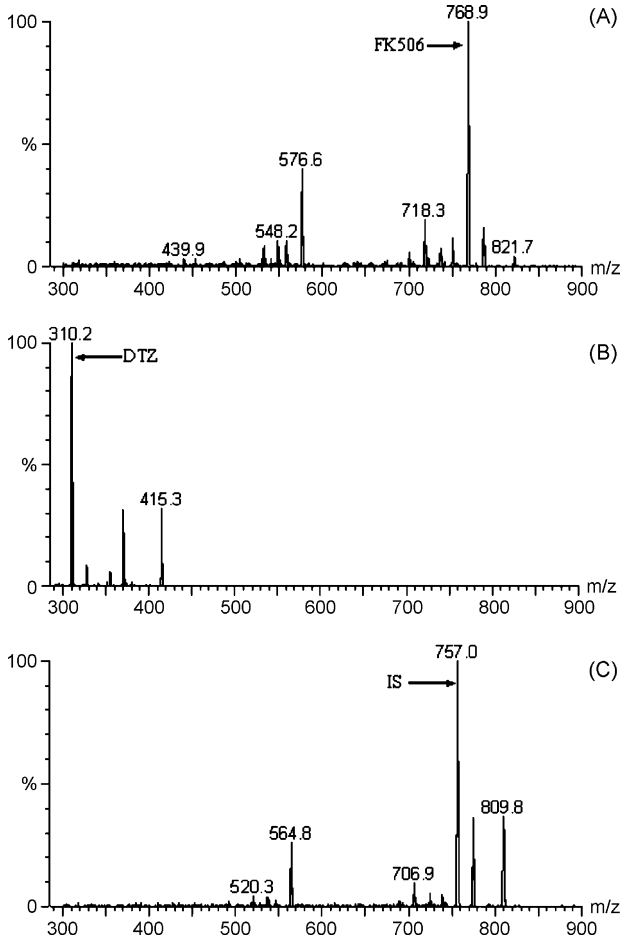

Fig. 2. Representative full-scan product ion spectra of the ammonium-adduct ion molecules of tacrolimus (FK506, A) and ascomycin (FK520, IS, C), and protonated molecule of diltiazem (DTZ, B).

samples at LLOQ levels. Endogenous peaks at the retention time of the analytes were not observed for any of the whole blood batches evaluated. This indicated no significant direct interference in the MRM channel for the analytes at the expected retention time. Representative chromatograms of blank human whole blood and the whole blood with added FK506 and DTZ at LLOQ levels are shown in Fig. 3. The retention times for FK506, DTZ and IS were 1.17, 1.16 and 1.08 min, respectively. The method had comparable and significantly shorter running time for determination of FK506 and DTZ, respectively, compared with those reported in the literature [29–33].

3.2.2. Matrix effects

An ME occurs when a biological sample contains a component that does not give a signal in the MRM channel used for the target analyte but co-elutes with the analyte and affects the spectral response of the analyte [34]. The presence of an ME can decrease or increase the response of the analyte and thus affect the sensitivity of a developed method. The result of ME at QC concentrations of FK506 and DTZ and at working concentration of IS in six different batches of human whole blood shows that there was an ME as indicated by values of <100% (ranging from 86.7–90.5%, 51.4–63.4% and 92.7–95.5% for FK506, DTZ and IS, respectively) in the area in spiked whole blood samples post-extraction. This demonstrated an ionization suppression effect for FK506 and DTZ under the present chromatographic and extraction conditions when an ESI interface was employed. Notably, the ionization suppression for the analytes observed kept consistent over the QC concentration ranges of the analyte without showing any analyte concentration-dependence. The ion suppression of IS was minimal. The RSD (%) of ME at different concentrations of FK506, DTZ and IS in six different lots of human whole blood was considered to be the assessment of the relative ME effect. The variability was acceptable, with RSD values <13.7% and <12.2% at different concentrations of FK506 and DTZ, respectively, and <3% at working concentration of IS, indicating that the relative ME for the analytes was minimal in this study.

Moreover, such ionization suppression did not affect the slopes and linearity of the established calibration curves, the ratio of analyte over IS, and precision and accuracy data throughout the analytical period. Thus, the present analytical method was considered reliable with a high sensitivity for FK506 and DTZ determination in human whole blood, in spite of the matrix effects existed.

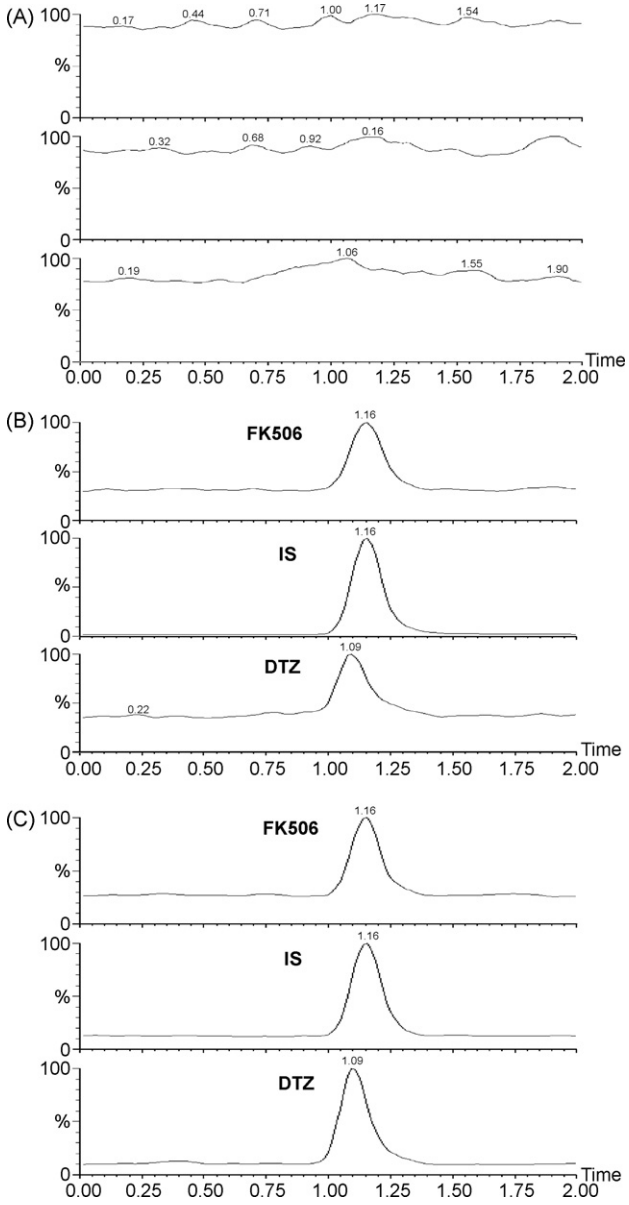

Fig. 3. Representative multiple reaction monitoring (MRM) chromatograms of tacrolimus (FK506), diltiazem (DTZ) and ascomycin (FK520, IS) in human whole blood. (A) Blank whole blood sample; (B) whole blood sample with added FK506 and DTZ at an LLOQ level (0.5 ng/mL and 2 ng/mL, respectively) and (C) a whole blood sample from a patient 12 h after dosing.

Table 2
Slope, intercept and correlation coefficient (r) for the calibration curves for FK506 and diltiazem (DTZ) ($n = 5$)

Compound	Concentration range (ng/mL)	Slope[a]		Intercept[a]		r^2
		Mean ± SD	CV[b] (%)	Mean ± SD	CV[b](%)	
FK506	0.5–200	0.0870 ± 0.0046	5.3	0.0141 ± 0.0014	9.9	≥0.995
DTZ	2–250	0.0125 ± 0.0008	6.1	0.0083 ± 0.0007	7.9	≥0.995

[a] Slope and intercept were determined automatically by Waters Masslynx software.
[b] CV, coefficient of variation = SD/Mean × 100.

Table 3
Intra- and inter-day precision and accuracy data for assays of tacrolimus (FK506) and diltiazem (DTZ) in human whole blood ($n = 5$)

Compound	Nominal concentration (ng/mL)	Precision		Accuracy (mean RSD, %)
		Mean ± SD	CV (%)	
Intra-day				
FK506	2	1.9 ± 0.0	2.6	−4.9
	10	10.0 ± 0.2	2.0	0.2
	50	49.3 ± 1.4	2.9	−0.5
DTZ	5	5.0 ± 0.2	4.8	−0.3
	25	25.8 ± 1.1	4.2	3.2
	100	99.0 ± 5.3	5.3	−1.0
Inter-day				
FK506	2	2.0 ± 0.2	9.6	3.4
	10	10.1 ± 0.5	5.0	1.1
	50	49.0 ± 2.3	4.7	0.5
DTZ	5	4.8 ± 0.1	2.3	−4.1
	25	25.3 ± 0.9	3.7	1.3
	100	96.6 ± 6.2	6.4	−3.4

CV, coefficient of variation = SD/Mean × 100.

In addition, the "cross-talk" between the three MS/MS channels used for monitoring was assessed by separately injecting FK506, DTZ and IS and monitoring the response in the other two channels. No "cross-talk" between channels was observed.

3.2.3. Linearity and lower limit of quantification

The linear regression analysis of FK506 or DTZ was constructed by plotting the peak-area ratio of theses two compounds to the internal standard (Y) versus analyte concentration (ng/ml) in spiked whole blood (X). The calibration curves were constructed in the range 0.5–200 ng/ml for FK506 and 2–250 ng/ml for DTZ. The calibration model for the calibration curve could be achieved by a linear equation of $Y = aX + b$ and $1/X$ and $1/X^2$ weighting factor for FK506 and DTZ, respectively. The slope, the intercept and the correlation coefficient (r^2) for each standard curve from each analytical run were determined automatically by the Waters Masslynx V 4.0 software program, and the data are summarized in Table 2. The regression equation of these curves and their correlation coefficients (r^2) were calculated as follows: FK506, $Y = 0.0870X + 0.0141$ ($r^2 = 0.9994$); DTZ, $Y = 0.0125X + 0.0083$ ($r^2 = 0.9973$); it showed good linear relationships between the peak areas and the concentrations. The lowest concentration on the calibration curve of FK506 and DTZ was 0.5 and 2 ng/mL, respectively. The analytes' response at these concentration levels was >5 times of the baseline noise. The precision and accuracy at these concentration levels were acceptable, with <5.5% CVs and <3.3% relative errors for FK506, and <4.2% CVs and <1.3% relative errors for DTZ. Thus, the lowest concentration on the calibration curve was accepted as the LLOQ. The LOD value was 0.1 ng/mL for FK506 and 0.5 ng/mL for DTZ.

3.2.4. Accuracy and precision

Intra- and inter-precision was assessed from the results of QC samples. The intra- and inter-day precision and accuracy data for FK506 and DTZ are summarized in Table 3. The reproducibility of the method was defined by examining both intra- and inter-day variance. For FK506 and DTZ, the intra-day precision ranged over 2.0–2.6% and 4.2–5.3%, and the inter-day precision was 4.7–9.6% and 2.3–6.4%. The mean intra-day errors were −4.6–0.2% and −1.0–3.2%, and the mean inter-day errors were 0.5–3.4% and −4.1–1.3% for the two compounds, respectively. The results above demonstrated that the values were within the acceptable range and the method was accurate and precise.

3.2.5. Recovery and stability

The recovery (extraction efficiency) of FK506 and DTZ from human whole blood following ethyl acetate extraction ranged over 58.3–62.6% and 50.4–58.4%, respectively; and were similar at all analyte concentrations without significant concentration dependence (Table 4). This indicated that the extraction efficiency for FK506 and DTZ was acceptable. There was no significant degradation under the conditions described in this study, since their concentrations deviated by no more than 13.1% relative to the reference nominal concentrations. All stability results are shown in

Table 4
The recovery (extraction efficiency) for tacrolimus (FK506) and diltiazem (DTZ) in human whole blood ($n = 5$)

Compound	Nominal concentration (ng/mL)	Recovery[a]	
		Mean ± SD	RSD (%)
FK506	2	58.7 ± 2.4	4.1
	10	62.6 ± 5.8	11
	50	58.3 ± 4.4	7.6
DTZ	5	50.4 ± 6.5	12.8
	25	54.8 ± 4.5	10.2
	100	58.8 ± 2.4	4.9

RSD, relative standard deviation.
[a] The recovery (extraction efficiency) of analytes from human whole blood after the extraction procedure was determined by comparing the areas of extracted analytes with that of the non-extracted pure standards that represent 100% recovery.

Table 5
Stability of tacrolimus (FK506) and diltiazem (DTZ) in human whole blood under various storage conditions ($n = 5$)

Storage condition	Compound	Nominal concentration (ng/mL)	Calculated concentration	
			Mean ± SD	Relative error[a] (%)
−20 °C/1 month				
	FK506	2	2.1 ± 0.2	5.0
		10	10.5 ± 0.5	4.7
		50	53.9 ± 1.4	7.7
	DTZ	5	5.6 ± 0.8	12.0
		25	28.03 ± 2.3	12.1
		100	107.3 ± 6.4	7.3
−20 °C/3 freeze-thaw cycles				
	FK506	2	1.8 ± 0.1	8.0
		10	11.3 ± 0.9	13.1
		50	54.2 ± 3.4	8.3
	DTZ	5	4.7 ± 0.3	−6.4
		25	27.5 ± 2.0	10.1
		100	111.6 ± 5.8	11.6
4 h at room temperature				
	FK506	2	2.0 ± 0.1	−2.5
		10	10.8 ± 0.4	8.1
		50	53.3 ± 1.4	6.5
	DTZ	5	5.0 ± 0.5	0.2
		25	27.9 ± 1.0	11.6
		100	106.6 ± 3.9	6.6
24 h at room temperature (extracted samples)				
	FK506	2	2.0 ± 0.16	1.5
		10	11.1 ± 0.4	10.5
		50	50.5 ± 1.5	1.1
	DTZ	5	4.6 ± 0.3	−7.6
		25	26.9 ± 1.1	7.5
		100	97.2 ± 7.2	−2.8

[a] Relative error: (overall mean assayed concentration-added concentration)/added concentration × 100.

Table 5. Both FK506 and DTZ in human whole blood can therefore be stored at room temperature for 4 h, 1 month at −20 °C and after three freeze-thaw cycles. Analysis of the QC samples following ethyl acetate extraction procedure showed no significant degradation after 24 h at room temperature. These results indicated that both FK506 and DTZ were stable under routine laboratory conditions and no specific procedure (e.g., acidification or addition or organic solvents) was needed to stabilize the compounds for daily clinical drug monitoring and pharmacokinetic study.

3.3. Application to pharmacokinetic study

The pharmacokinetic profiles of FK506 with and without coadministration of DTZ were investigated by the developed method in renal transplantation patients. The mean whole blood concentration-time curves of FK506 and DTZ among the six

Table 6
Pharmacokinetic parameters of tacrolimus with and without coadministration of diltiazem in six renal transplantation patients (mean ± SD, $n = 6$)

Parameter	FK506 alone	FK506 + DTZ
C_0 (ng/mL)	5.5 ± 1.1	6.0 ± 1.0
C_{max} (ng/mL)	11.7 ± 1.6	14.1 ± 0.8*
T_{max} (h)	1.7 ± 0.7	1.8 ± 1.2
$AUC_{0-12\,h}$ (ng/mL h)	85.5 ± 13.6	105.5 ± 15.5*

Data are the mean ± SD. Abbreviations: $AUC_{0-12\,h}$, area under the plasma concentration-time curve from time zero to the last measurable time point (12 h); C_{max}, maximum whole blood concentration; FK506, tacrolimus; DTZ, diltiazem; T_{max}, maximum time to reach C_{max}; C_0, whole blood concentration of FK506 and DTZ before they were administrated.

* $P < 0.05$, by Wilcoxon Signed-Rank Test.

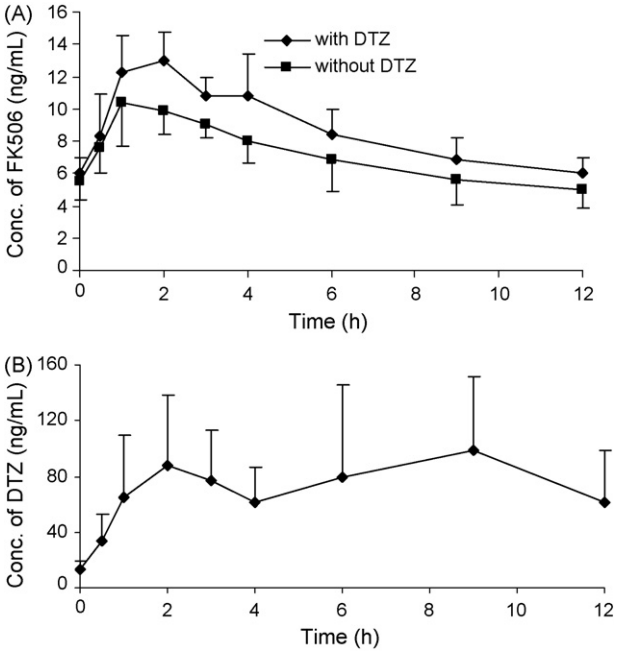

Fig. 4. The mean steady-state concentration-time profiles of tacrolimus (FK506, A) in six renal transplantation patients when used alone or in combination with diltiazem (DTZ). Plot B illustrates the steady-state pharmacokinetic profile of DTZ in these patients (mean ± SD, $n = 6$). FK506 at 0.065 mg/kg was orally administrated twice a day, and 30 mg DTZ was orally taken three times a day for two weeks.

Table 7
The steady-state pharmacokinetic parameters of diltiazem (DTZ) in six renal transplantation patients

No. of subject	C_0 (mg/L)		C_{max} (mg/L)		$AUC_{0\text{-}t}$ (mg/L h)	
	0 h	4 h	0–4 h	4–12 h	0–4 h	4–12 h
1	12.19	42.75	49.9	93.6	172.2	587.5
2	13.28	27.96	43.5	35.3	126.0	197.1
3	11.3	36.36	120.9	88.7	309.1	495.9
4	24.71	104.14	173.9	206.5	500.6	1358.9
5	8.04	69.04	111.8	112.0	251.2	735.9
6	9.37	40.76	78.5	98.0	229.0	526.9
Mean ± SD	13.1 ± 6.0	53.5 ± 28.4	96.4 ± 49.3	105.7 ± 55.9	264.7 ± 131.8	650.4 ± 389.3

patients were shown in Fig. 4, and the calculated pharmacokinetic parameters of FK506 with and without DTZ were presented in Table 6. Without coadministration of DTZ, the mean C_{max} and $AUC_{0-12\,h}$ of FK506 were 11.7 ± 1.6 ng/mL and 85.5 ± 13.6 ng/mL h, respectively. When used in combination with DTZ, the mean C_{max} and $AUC_{0-12\,h}$ of FK506 were increased to 14.1 ± 0.8 ng/mL and 105.5 ± 15.5 ng/mL h, respectively ($P < 0.05$). However, there was no significant difference in C_0 and T_{max} for FK506 before and after coadministration with DTZ. The steady-state plasma concentration-time profiles of DTZ are shown in Fig. 4 and the pharmacokinetic parameters are shown in Table 7. Notably, there were remarkable twin peaks for DTZ following oral administration, with first peak appearing gat 2 h and second peak at 9 h post-dosing. There was a significant inter-individual variability in the C_{max} and AUC values of DTZ (4.0–6.9 folds). These results indicate that DTZ significantly increased the C_{max} and $AUC_{0-12\,h}$ of FK506, probably due to inhibition of CYP3A-mediated first-pass metabolism of FK506 and enhanced intestinal absorption due to inhibition of P-glycoprotein. DTS does not appear to change the absorption rate of FK506. Further investigation about the effect of DTZ on the FK506 pharmacokinetics and pharmacodynamics is warranted.

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