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Article

Rapid and Sensitive UPLC–MS-MS Determination of Tacrolimus in Wistar Rats and Human Blood

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A simple, sensitive and high throughput ultra-high performance liquid chromatography tandem mass spectrometry (UPLC–MS-MS) method was developed for the determination of tacrolimus in the whole blood of Wistar rats and humans. Sample preparation involved protein precipitation of the analyte, using sirolimus as the internal standard with $ZnSO_4$ from 50 μL of rat blood/human blood, followed by solid-phase extraction. Chromatographic analysis was conducted on a Waters Acquity UPLC BEH C18 column (50 × 2.1 mm, 1.7 μm) using 10 mM ammonium acetate (pH 6.0) and methanol (5:95, v/v) under isocratic conditions and detection by MS-MS. Quantitation of the analytes was achieved by multiple reaction monitoring under positive ionization mode. The method was validated over a dynamic concentration range of 0.200–200 ng/mL and had a chromatographic run time of 1.2 min. The extraction recovery for tacrolimus was >96% across three quality control levels. Matrix effect was assessed by the precision (coefficient of variation) values for the calculated slopes of calibration curves from six lots of blood. The method was applied to a pre-clinical study in 25 rats and to a bioequivalence study in 20 healthy Indian subjects. The reproducibility of the assay was successfully demonstrated by the reanalysis of 80 subject samples.

Introduction

Tacrolimus (FK-506) is a macrolide lactone with potent immunosuppressive properties that is produced by the fungus *Streptomyces tsukubaensis*. It is used primarily to prevent the rejection of allogenic solid organ transplants in recipients (1, 2). Initially, it was employed in the management of liver transplants; it is now routinely used in the management of kidney, heart, pancreas, small bowel, lung and bone marrow transplants (3, 4). FK-506 acts by binding to a cytoplasmic protein called immunophilin; the resultant complex then inhibits the function of an intracellular protein calcineurin, a Ca and calmodulin-dependent serine/threonine phosphatase. Furthermore, this interaction leads to the inhibition of T-lymphocyte signal transduction and decreases IL-2 transcription, which gives rise to immune suppression (3–5). Because of the variability in the absorption and clearance of FK-506 and the very low levels found in plasma, monitoring of the drug in whole blood is recommended to achieve optimal therapeutic efficacy while minimizing the risk of toxicity (3). FK-506 is a critical dose drug with a narrow therapeutic index; i.e., it exhibits the desired therapeutic effect with acceptable tolerability within a narrow range of blood concentration. As a result, at low blood levels there is a risk of rejection of the organ transplant, while elevated circulating concentration can lead to serious toxicity and long-term morbidity (6, 7). Furthermore, FK-506 exhibits a high degree of inter- and intra-individual pharmacokinetic and dynamic variability, which increases the risk of therapeutic failure if used at a uniform dose in all patients. Thus, the accurate determination of FK-506 is essential to correlate its blood concentration and clinical outcomes for therapeutic drug monitoring (8–10). Currently, there are two approaches for the measurement of FK-506; namely, immunoassays (11–15) and liquid chromatography–tandem mass spectrometry (LC–MS-MS) methods. Immunoassays are widely used for the routine determination of FK-506; however, they lack specificity due to endogenous compounds and cross reactivity of monoclonal antibodies with the metabolites of the drug (13, 16). On the other hand, LC–MS-MS based methods are highly selective because they depend on the physicochemical properties of the drug for detection and quantitation (17). Although there are several LC–MS-MS methods reported in literature to determine FK-506, either alone (18–25) or with other immunosuppressant drugs (26–42) in human whole blood, very few studies have analyzed the drug in rat whole blood samples (43, 44). Babu *et al.* (43) determined FK-506 in rat whole blood using triple quadrupole LC–MS with a sensitivity of 20.9 ng/mL. However, the application of the method in real samples was not shown. A microparticle enzyme immunoassay (MEIA) has been proposed for the determination of FK-506 in the whole blood of Sprague-Dawley rats (44). The assay was calibrated from 3.0–30.0 ng/mL, and the lower limit of detection was 1.5 ng/mL. An ultra-fast liquid chromatography–tandem mass spectrometry method has also been reported for the simultaneous determination of FK-506, cyclosporine A, sirolimus and everolimus in human whole blood (45). The method was validated in the calibration range of 1.0–44 ng/mL for FK-506 using a sample volume of 100 μL for processing.

Ultra-performance liquid chromatography (UPLC) can serve as a superior alternative to high-performance liquid chromatography (HPLC), especially in reducing the analysis time when large numbers of samples are to be analyzed in a clinical setting. This technology is capable of achieving higher peak capacity, speed and sensitivity than conventional HPLC. In addition, solvent consumption can be considerably reduced compared to conventional 4.6 mm i.d. columns (46). Thus, in the present work, an accurate, simple, sensitive and rapid UPLC–MS-MS method has been developed and fully validated for the

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reliable measurement of FK-506 in healthy human volunteers and in rat blood samples for a preclinical study. The method requires only a 50 μL rat/human blood sample for extraction and demonstrates excellent performance in terms of ruggedness and chromatographic efficiency (1.2 min per sample). Ion-suppression effects were investigated by post-column infusion of the analyte. It was successfully applied to a pharmacokinetic study of five FK-506 formulations in 25 Wistar rats. The method was also validated in human blood and was used to support a pilot bioequivalence study on 20 healthy human subjects.

Experimental

Chemicals and materials

Reference standards of FK-506 (99.5%) and sirolimus [internal standard (IS), 99.4%] were obtained from Biocon Ltd. (Bangalore, India). HPLC-grade methanol was procured from Merck (Darmstadt, Germany). Formic acid, zinc sulfate and ammonium acetate were purchased from Spectrochem (Mumbai, India). Deionized water was prepared using Milli-Q water purification system from Millipore (Bangalore, India). A LiChrosep Sequence extraction cartridge (30 mg, 1 cc) was purchased from Merck Specialties (Mumbai, India). Blank rat blood was obtained from an in-house facility, and blank human blood was obtained from an in-house clinical department and were stored at −20°C until use.

Liquid chromatographic and mass spectrometric settings

Chromatographic analysis was conducted on a Waters Acquity UPLC system (Milford, MA) equipped with Acquity UPLC BEH C18 analytical column (50 × 2.1 mm, 1.7 μm) that was maintained at 35°C in a column oven. The mobile phase consisted of 10 mM ammonium acetate, pH 6.00, adjusted with formic acid and methanol (5:95, v/v), and was delivered at a flow rate of 0.350 mL/min. Ionization and detection of FK-506 and IS were conducted on a Quattro Premier XETM mass spectrometer from Waters Micro Mass Technologies, equipped with a turbo ion spray interface and operated in positive ionization mode. Multiple reaction monitoring (MRM), using precursor → product ion transitions of m/z 821.3 → 768.3 and m/z 931.5 → 864.6, was used to quantify FK-506 and IS, respectively. The source-dependent parameters maintained for FK-506 and IS were as follows: desolvation gas, 700 L/h; capillary voltage, 3.50 kV; desolvation temperature, 400°C; entrance potential, 1.0 V; and cone gas flow, 50 L/h. The optimum values for compound-dependent parameters like cone voltage and collision energy were 35 and 22 eV for FK-506 and 33 and 22 eV for IS, respectively. Quadrupoles 1 and 3 were maintained at unit mass resolution and the dwell time was set at 100 ms for both drugs. Data collection, peak integration and calculations were performed using Mass Lynx software, version 4.1.

Standard stock solutions, calibration standards and quality control samples

The stock solution of FK-506 (1,000 μg/mL) was prepared by dissolving the accurately weighted reference standard in methanol. Calibration standards and quality control (QC) samples were prepared by spiking 49 μL of blank rat/human blood with 1 μL of the appropriate stock/intermediate solutions. Calibration curve standards were made at concentrations of 0.20, 0.40, 1.00, 4.00, 10.0, 20.0, 40.0, 100.0 200.0 ng/mL, whereas high, medium and low QC samples were prepared at concentrations of 160.0, 80.0 and 0.60 ng/mL, respectively. A stock solution (1.0 mg/mL) of the IS was prepared by dissolving the accurately weighted reference standard of sirolimus in methanol. Its working solution (500 ng/mL) was prepared by appropriate dilution of the stock solution in methanol. All solutions (standard stock, calibration standards and QC samples) were stored at 5°C until use.

Blood sample preparation

All frozen subject samples, calibration standards and quality control samples (in K_2EDTA) were thawed and allowed to equilibrate at room temperature before extraction. To an aliquot of 50 μL of spiked blood sample, 10 μL of IS was added and vortex-mixed for 15 s. Furthermore, the mixture was pretreated with 50 μL of 0.1M zinc sulphate solution in water to lyse the cells, followed by vortex-mixing for 1.0 min. After centrifugation for 2 min at 1,811 × g, the supernatant was separated and subjected to solid-phase extraction (SPE) on a Lichrosep Sequence cartridge (with functionalized polydivinylbenzene as the sorbent, average pore size of 200 Å and surface area of 794 m^2/g), which was previously equilibrated with 1.0 mL of methanol followed by 1.0 mL of water. The cartridge was washed with 2.0 mL of water, followed by elution of the analyte and IS with 0.5 mL methanol in pre-labeled RIA vials. The solvent was then evaporated to dryness in a thermostatically controlled water bath maintained at 40°C under a gentle stream of nitrogen. The dried sample was reconstituted in 100 μL of the mobile phase and 10 μL was used for injection into the chromatographic system.

Method validation procedures

The method was validated for rat and human blood following the United States Food and Drug Administration (FDA) guidelines (47). A system suitability experiment was performed by injecting six consecutive injections using aqueous standard mixtures of FK-506 (80.0 ng/mL) and IS (500 ng/mL) at the start of each batch during method validation. The performance of the system was studied by injecting one extracted lower limit of quantitation (LLOQ) sample with IS at the beginning of each analytical batch and before re-injecting any batch during method validation. The carryover effect of the autosampler was evaluated by sequentially injecting extracted blank blood → upper limit of quantitation (ULOQ) sample (highest concentration in the calibration curve) → extracted blank blood → LLOQ sample (lowest concentration in the calibration curve) → extracted blank blood.

The selectivity of the method toward endogenous matrix components was assessed in six different batches of blank blood. The linearity of the method was determined by the analysis of four linearity curves containing nine non-zero concentrations. The area ratio response for FK-506/IS obtained from MRM was used for regression analysis. Each calibration line was

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analyzed by least square weighted ($1/x^2$) linear regression. The lowest standard on the calibration line was accepted as the LLOQ, having at least 10 times more response than drug-free (blank) extracted blood.

To determine the intra-batch accuracy and precision, six replicates of QC samples (high, medium and low) along with calibration curve standards were analyzed on the same day. The inter-batch accuracy and precision were assessed by analyzing five precision and accuracy batches on three consecutive days. The precision [coefficient of variation (CV)] at each concentration level from the nominal concentration should not be greater than 15% and the accuracy should be within ±15%.

A post-column analyte infusion experiment for ion suppression/enhancement was conducted by infusing a standard solution of FK-506 (160 ng/mL) and IS into the mobile phase at 10 μL/min by employing an infusion pump. Aliquots of 10 μL of extracted control blood were then injected into the column by the autosampler and MRM chromatograms were acquired for FK-506 and IS.

The extraction recovery for FK-506 and IS was calculated by comparing the mean area response of extracted samples (spiked before extraction) to that of unextracted samples (spiked after extraction) at each QC level. The absolute matrix effect was assessed by comparing the mean area response of unextracted samples (spiked after extraction) with the mean area of standard solutions prepared in mobile phase. The assessment of relative matrix effect was based on the calculation of precision (CV) values for slopes of calibration lines from six lots of blank blood. For a method to be practically free from relative matrix effect, the CV should not exceed 3–4% (48).

The stability results were evaluated by measuring the area ratio responses (FK-506/IS) of stability samples against freshly prepared comparison samples with identical concentration. Stock solutions of FK-506 and IS were checked for short-term stability at room temperature and long-term stability at 5°C. Autosampler (wet extract), bench top (at room temperature), freeze-thaw and long-term stability in blood were performed at high and low QC levels using six replicates. The samples were considered stable if the deviation from the mean calculated concentration of freshly thawed QC samples was within ±15.0%.

The ruggedness of the method was evaluated on two precision and accuracy batches. The first batch was analyzed by different analysts and the second batch was studied on two different columns (same make but different batch number). The dilution reliability was determined by diluting the stock solution prepared as a spiked standard at a concentration of 600.0 ng/mL of FK-506 in the screened blood lot. The precision and accuracy for dilution integrity standards at 1/5th and 1/10th dilution were determined by analyzing the samples against freshly prepared calibration curve standards.

Pharmacokinetic study in Wistar rats and healthy Indian volunteers

The pre-clinical pharmacokinetic study was conducted on 25 male Wistar rats (8–10 weeks old) with mean ± standard deviation (SD) body weights of 350 ± 21 g at the time of use. The study was conducted strictly in accordance with the guidelines laid down by the Organization for Economic Co-operation and Development (OECD) for the testing of chemicals. The rats were evenly allocated into five groups (Groups A–E, five animals per group). After overnight fasting, each rat in Groups A–E was orally administered 1, 2, 3, 4 and 5 mg/kg, respectively, of FK-506 formulation. Venous blood of approximately 0.2 mL was collected from the bottom of the eye in K_2EDTA before dosing (0.0 h) and at 0.25, 0.50, 1.0, 2.0, 4.0 and 8.0 h post-dosing, and kept frozen at −20°C until analysis.

The design of the clinical study in healthy volunteers was a randomized, single dose, open label, bioequivalence study of test (5 mg FK-506 capsules from Indian Pharmaceuticals Company, India) and reference (Prograf, 5 mg FK506 capsules from Astellas Ireland Company, Kerry, Ireland) formulations in 20 healthy adult subjects (18–45 years) under fasting conditions. Written consent was taken from all subjects after informing them about the objectives and possible risks involved in the study. The study was conducted in accordance with International Conference on Harmonization and FDA guidelines (49). An incurred sample re-analysis was also conducted by the selection of 80 human samples (10% of total analyzed samples) near the maximum blood concentration (C_{max}) and the elimination phase in the pharmacokinetic profile of the drug. The percent change in the value should not be more than ±20% (50).

Results and Discussion

Method development

The present study was conducted using electrospray ionization (ESI) for MRM UPLC–MS-MS analyses to attain high sensitivity and linearity in regression curves. Initially, ionization of the analyte and IS was attempted in the negative and positive modes to achieve maximum sensitivity. Although the deprotonated precursor ion was detected in the negative mode, the sensitively was not adequate to detect the required LLOQ of 0.2 ng/mL. As reported previously (20), in the positive ionization mode, the protonated precursor ions for the analyte had very low abundance due to weak affinity for protons. The protonated ion $[M+H]^+$ was practically undetected in the positive Q1 ESI scan. However, the analyte showed strong ability to bind with sodium and potassium ions to form $[M+Na]^+$ and $[M+K]^+$ singly charged adducts, and had peaks corresponding to m/z 826.2 and 842.6, respectively. Although sodium or potassium adduct ions are readily formed in the ion source, their fragmentation is relatively difficult. Thus, ammonium acetate was used in the mobile phase to form ammonium adduct ions, which have relatively higher abundance and can be easily fragmented for MS-MS detection. The most abundant precursor ions (ammonium adducts) were observed at m/z 821.3 and 931.5 for the analyte and IS, respectively, in the Q1 MS full scan spectra. The most abundant product ions in the Q3 MS spectra for FK-506 and IS were found at m/z 768.3 and 864.6, respectively, at collision energy of 22 eV (Supplementary Figure 1). The fragment at m/z 768.3 corresponds to the elimination of two water molecules and ammonia from the precursor ion of FK-506, whereas the product ions at m/z 864.6 can be attributed to the removal of methanol, ammonia and a water

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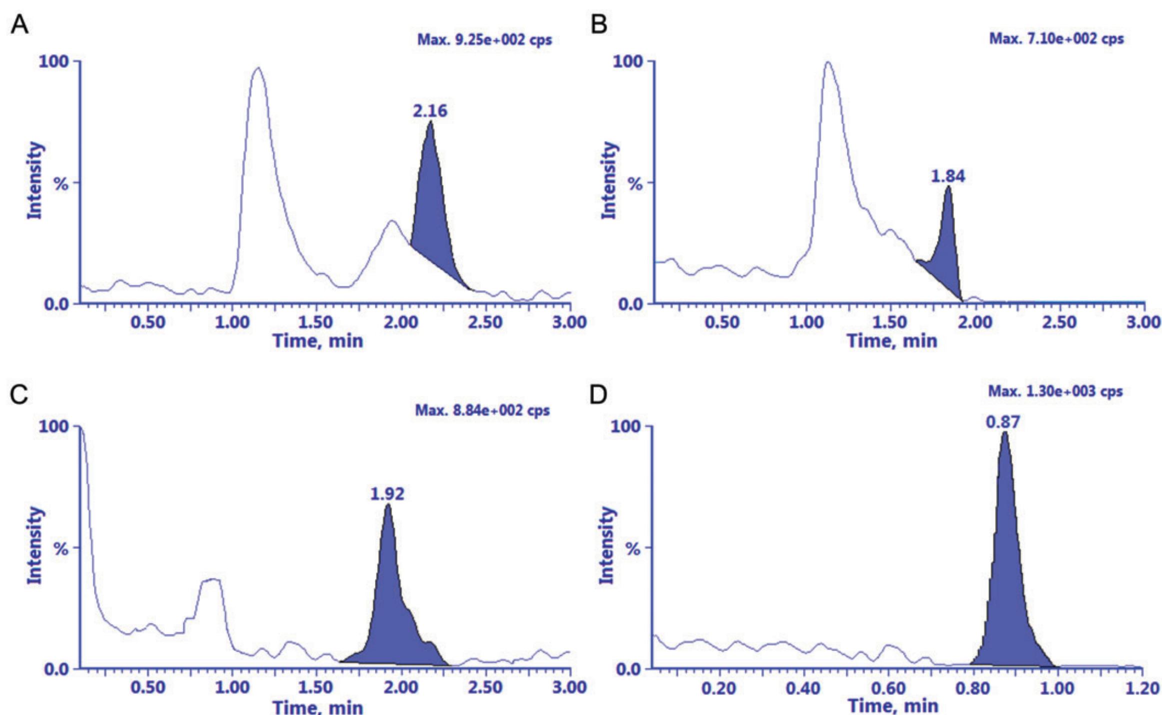

Figure 1. Chromatograms for FK-506 (m/z 821.3 → 768.3) at LLOQ level (extracted sample) using 10 mM ammonium acetate as the mobile phase, pH 6.00, adjusted with formic acid and methanol (5:95, v/v) on: Gemini NX C18 (100 × 4.6 mm, 5 μm) (A); Kromasil Grace C18 (50 × 4.6 mm, 5 μm) (B); Hypersil Gold C18 (50 × 4.6 mm, 5 μm) (C); Waters Acquity UPLC BEH C18 (50 × 2.1 mm, 1.7 μm) (D). Injection volume: 10 μL.

molecule from the sirolimus precursor ion. All other product ions observed in the Q3 MS of FK-506 (m/z 786.7, 768.3, 718.6 and 576.8) and sirolimus (m/z 896.4, 864.6 and 846.5) were either inconsistent or gave low responses, and hence, were not considered in the present work. Furthermore, the source-dependent and compound-dependent parameters were suitably optimized to obtain consistent and adequate responses for the analyte and IS.

As evident from the literature, very few methods have been described for the analysis of FK-506 from rat blood (43, 44). Babu *et al.* (43) employed liquid–liquid extraction (LLE) with methyl *tert*-butyl ether to extract FK-506 from 100 μL of rat blood, with a mean recovery of only 23.92%. However, the majority of the methods have used protein precipitation (PP) (35, 36, 38, 41), a combination of PP and SPE (18, 22, 25, 26, 31, 37, 39) or PP and LLE (20, 24) for quantitative and consistent recoveries of FK-506 from human blood. Because FK-506 is sequestered within the erythrocytes, it is essential to lyse the cells with a protein precipitant to free the analyte. In the present work, $ZnSO_4$ was used as the protein precipitant, as reported previously (25, 26), followed by SPE on LiChrosep Sequence extraction cartridges employing a rat blood sample of 50 μL. Washing the cartridges with 2.0 mL of water was adequate to remove endogenous compounds. Quantitative recovery was possible using 0.5 mL of methanol for elution at all QC levels.

Initially, during development, different analytical columns were tested to produce a short run time, good peak shapes and minimum matrix interference and solvent consumption. Because several columns of different dimensions and particle sizes have been used in reported methods to analyze FK-506 (18, 20, 22–33, 35–43, 45), four different columns were tested in the present work. These included Gemini NX C18 (100 × 4.6 mm, 5 μm), Kromasil Grace C18 (50 × 4.6 mm, 5 μm), Hypersil Gold C18 (50 × 4.6 mm, 5 μm) and Waters Acquity UPLC BEH C18 (50 × 2.1 mm, 1.7 μm). Furthermore, the mobile phase was optimized using methanol–acetonitrile and ammonium acetate–formate and with acidic buffers like formic acid–ammonium formate and acetic acid–ammonium acetate in different compositions. The chromatography was poor on Gemini NX C18 and Kromasil Grace C18 columns, as shown in Figures 1A and B, respectively. The detector response on the Hypersil Gold C18 column (50 × 4.6 mm, 5 μm) was comparatively high, however, the peak shape was unacceptable (Figure 1C). Nevertheless, the best chromatographic conditions as functions of analyte peak intensity and peak shape and analysis run time was achieved with the Acquity UPLC BEH C18 column (50 × 2.1 mm, 1.7 μm) using 10 mM ammonium acetate as the mobile phase, pH 6.00, adjusted with formic acid and methanol (5:95, v/v) under isocratic conditions (Figure 1D). The total chromatographic run time was 1.2 min with a retention time of 0.87 min for FK-506. Representative MRM ion chromatograms, as shown in Figure 2, of extracted blank rat blood (double blank), blank blood fortified with IS, FK-506 at LLOQ and an actual rat sample at 2.0 h, demonstrate the selectivity of the method. Chromatograms for post-column infusion experiment, as shown in Figure 3, indicate no ion suppression or enhancement at the retention times of FK-506 and

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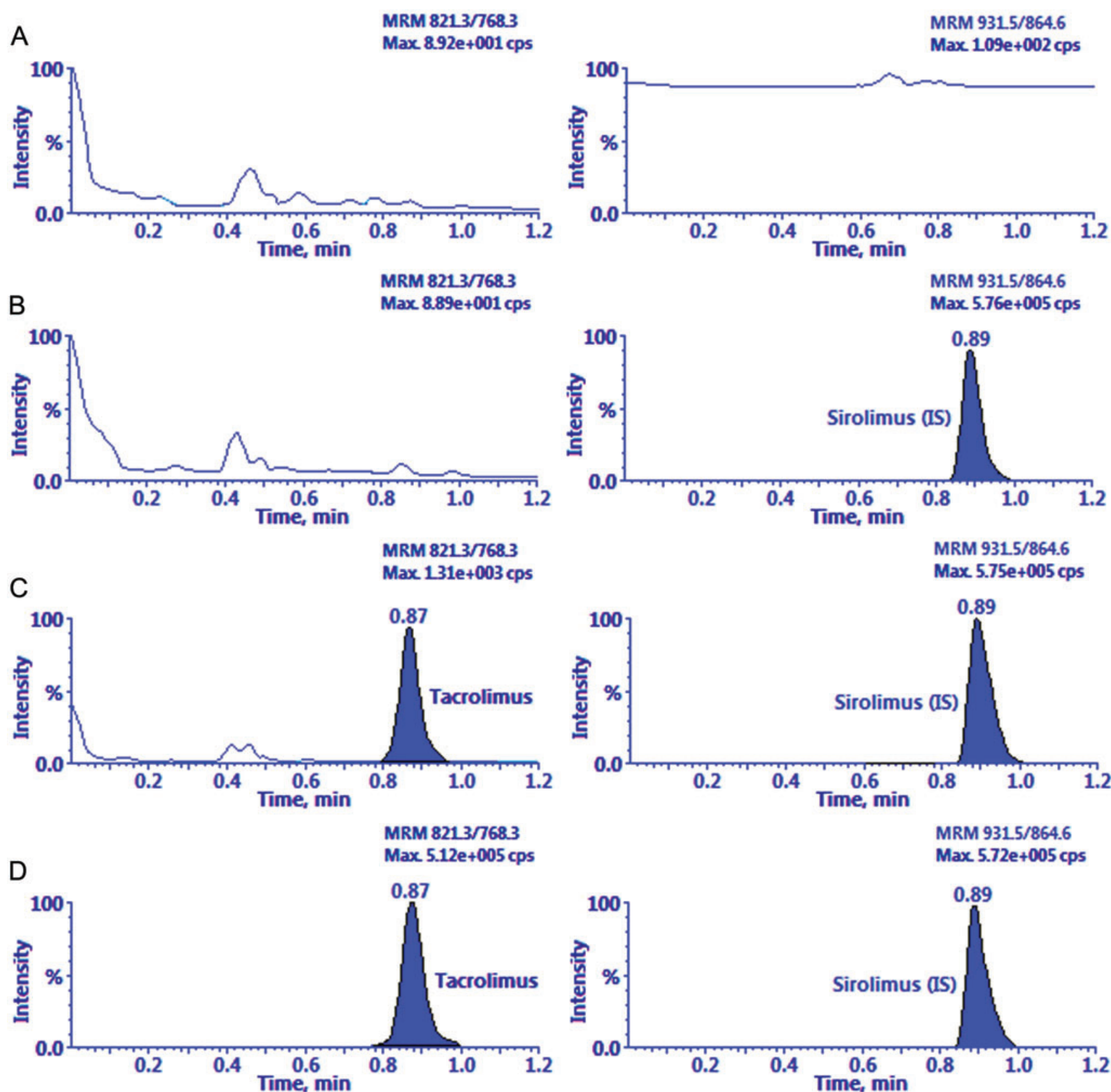

Figure 2. MRM ion-chromatograms: double blank blood (without IS) (A); blank blood with IS (m/z 931.5 → 864.6) (B) FK-506 (m/z 821.3 → 768.3) at LLOQ and IS (C); real rat blood sample at 2.0 h after administration of a 1.0 mg/kg dose of FK-506.

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IS. Sirolimus, used as an IS, is a macrocyclic lactone like FK-506. It has similar chromatographic behavior and was easily extracted with the proposed extraction protocol. There was no effect of IS on analyte recovery, sensitivity or ion suppression. The average matrix factor value, calculated as the response of the post-spiked sample/response of neat solutions in mobile phase at the HQC, MQC and LQC levels, was 0.97, which indicates a minor suppression of 3%.

Results for method validation

Because of the almost similar validation results in rat and human blood, only rat blood data has been presented to avoid duplication. The precision (CV) of the system suitability test was observed in the range of 0.04 to 0.11% for the retention time and 1.12 to 2.54% for the area response of FK-506 and IS. The signal-to-noise ratio for system performance was ≥60 for FK-506 and IS. Carryover evaluation was performed in each analytical run to ensure that it did not affect the accuracy and the precision of the proposed method. Practically negligible carryover (≤0.8%) was observed during the autosampler carryover experiment (Supplementary Figure 2).

The calibration lines were linear over the concentration range of 0.20–200.0 ng/mL. The mean linear equation was $y = (0.008167 \pm 0.00009)\ x + (0.00104 \pm 0.00014)$ for FK-506, where y is the peak area ratio of the analyte/IS and x is the concentration of the analyte. The mean ± SD value for correlation coefficient (r^2) was 0.9982 ± 0.00083 for FK-506. The accuracy and precision (CV) observed for the calibration curve standards ranged from 94.4 to 103.2% and 1.62 to 1.97, respectively. The lowest concentration in the standard curve that could be measured with acceptable accuracy and precision was 0.200 ng/mL at a signal-to-noise ratio (S/N) ≥ 60. Based on the high S/N values, it was possible to lower the quantitation limit

by 6 fold, however, this was not required based on subject sample results.

The intra-batch precision (CV) ranged from 3.74 to 4.93% and the accuracy was within 97.0 and 100.7%. For the inter-batch experiments, the precision varied from 3.31 to 4.42 and the accuracy was within 99.7 and 102.2% (Table I). The relative recovery, absolute matrix effect and process efficiency data at three QC levels are presented in Table II. The mean extraction recoveries were 97.1 and 100.8% for FK-506 and IS, respectively. The relative matrix effect, as recommended by Matuszewski *et al.* (48), offers a comparison of matrix effect values between different lots of blood. The CV of the slopes of calibration lines for relative matrix effects in six different blood lots was 1.03% for FK-506 (Supplementary Table I).

The stability of the FK-506 and IS in rat blood and stock solutions was examined under different storage conditions. Stock solutions of FK-506 and IS remained unchanged up to 9.0 h at room temperature (short-term stability) and for a minimum of 73 days at refrigerated temperature, 5°C (long-term stability). FK-506 in control rat blood (bench top) at room temperature was stable for a minimum period of 9.0 h at 25°C and five freeze and thaw cycles at −20°C. Spiked blood samples stored at −20°C for the long-term stability experiment were found to be stable for a minimum period of 72 days. The autosampler stability (wet extract) of the spiked quality control samples maintained at 5°C was determined up to 36.0 h without significant drug loss. All stability results in blood at two QC levels are shown in Table III.

For method ruggedness, the precision (CV) and accuracy values for FK-506 with two different columns ranged from 3.3 to 4.5% and 95.3 to 102.0%, respectively. For the experiment with different analysts, the results varied from 3.0 to 3.7% and 97.9 to 102.3% for precision and accuracy, respectively. The precision for dilution integrity of 1/5 and 1/10th dilution were 2.7 and 3.2%, and the accuracy results were 94.4 and 103.4%, respectively, which is well within the acceptance limits of 15% for precision (CV) and 85 to 115% for accuracy.

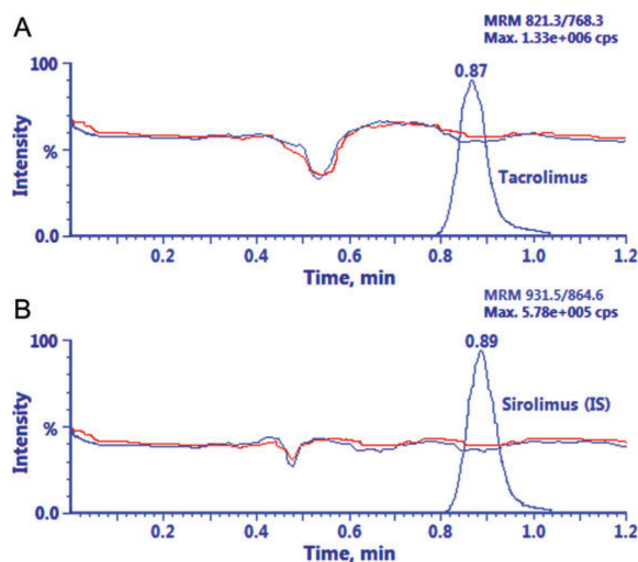

Figure 3. MRM LC–MS-MS chromatograms of blank blood with post column infusion of: FK-506 (A); sirolimus (IS) (B).

Application of the method

The mean blood concentration–time profiles of FK-506 for male Wistar rats in Groups A–E are presented in Figure 4A. The estimated pharmacokinetic parameters are compiled in Table IV. Good linear correlation was observed between values of the half-life of drug elimination during the terminal phase ($t_{1/2}$), area under the blood concentration–time curve from zero hour to 8 h (AUC_{0-8}) and area under the blood concentration–time curve from zero hour to infinity ($AUC_{0-inf.}$) values. The C_{max} values were also proportional to the dose strength from Groups A–D. The method was also applied to quantify FK-506 concentration in human blood samples for a bioequivalence study after oral administration of

Table II
Absolute Matrix Effect, Relative Recovery and Process Efficiency for FK-506 in Rat Blood*

A (CV) (%)	B (CV) (%)	C (CV) (%)	Absolute matrix effect (B/A) × 100	Relative recovery (C/B) × 100	Process efficiency (C/A) × 100
LQC					
0.0056 (5.4)	0.0055 (2.3)	0.0053 (3.7)	98.2 (96.1)*	96.4 (99.7)*	94.7 (95.8)*
MQC					
0.7395 (2.0)	0.6922 (3.4)	0.6783 (5.8)	93.6 (94.2)*	97.9 (100.8)*	91.6 (94.9)*
HQC					
1.4958 (5.7)	1.4210 (4.8)	1.3784 (5.1)	95.0 (92.5)*	97.0 (101.9)*	92.2 (94.3)*

*Note: A represents the mean area ratio response of six replicate samples prepared in mobile phase (neat samples); B represents the mean area ratio response of six replicate samples prepared by spiking in extracted blank blood; C represents the mean area ratio response of six replicate samples prepared by spiking before extraction.
*Values for IS are in parentheses.

Table I
Intra-Batch and Inter-Batch Precision and Accuracy for FK-506 in Rat Blood

QC	Nominal concentration (ng/mL)	Intra-batch				Inter-batch			
		n	Mean concentration found (ng/mL)*	Accuracy (%)	CV (%)	n	Mean concentration found (ng/mL)†	Accuracy (%)	CV (%)
HQC	160.0	6	161.1	100.7	3.74	30	162.3	101.4	3.31
MQC	80.0	6	79.2	99.0	4.05	30	79.8	99.7	4.38
LQC	0.600	6	0.582	97.0	4.93	30	0.613	102.2	4.42

*Mean of six replicates at each concentration.
†Mean of six replicates for five precision and accuracy batches.

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5 mg test and reference formulations of FK-506. Figure 4B shows the blood concentration versus time profile of FK-506 in healthy human subjects. The mean pharmacokinetic parameters obtained in the study are presented in Table V. The values for C_{max}, time point of maximum blood concentration (T_{max}), $t_{1/2}$, elimination rate constant (K_{el}), AUC from zero hour to 144 h (AUC_{0-144}) and $AUC_{0-inf.}$ were in close agreement with the work of Mathew *et al.* (51), which involved 109 Indian subjects. Furthermore, the ratios of mean log-transformed parameters and their 90% confidence intervals (CIs) varied from 100.6 to 105.6%, which is within the defined bioequivalence range of 80–125% (Supplementary Table II).

Out of 80 incurred samples studied, four samples showed a percentage change within 10–15%, 13 samples were within ±5–10% and the remaining 63 samples were between a change of 0 and ±5%. This authenticates the reproducibility of the proposed method (Supplementary Figure 3).

Table III
Stability of FK-506 in Rat Blood under Different Conditions ($n = 6$)*

Storage condition	Nominal concentration (ng/mL)	Mean stability samples ± SD	Change (%)
Bench top stability; 9 h			
HQC	160.0	159.2 ± 5.08	−0.50
LQC	0.600	0.613 ± 0.022	2.17
Wet extract stability; 36 h			
HQC	160.0	156.5 ± 6.44	−2.19
LQC	0.600	0.584 ± 0.033	−2.67
Freeze and thaw stability; 5 cycles, −20°C			
HQC	160.0	152.9 ± 7.36	−4.44
LQC	0.600	0.568 ± 0.056	−5.33
Long-term matrix stability; 72 days, −20 °C			
HQC	160.0	164.1 ± 5.99	2.56
LQC	0.600	0.585 ± 0.048	−2.50

*Note:

$$\%\text{Change} = \frac{\text{Mean stability samples} - \text{Mean comparison samples}}{\text{Mean comparison samples}} \times 100.$$

Comparison with reported methods

The present method has the shortest chromatographic run time compared to many methods developed for FK-506 in whole blood (20, 22, 24–32, 35–43). Similarly, the blood processing volume is much lower than several methods (18, 20, 22, 24–29, 31–33, 36–40, 43, 45) and identical to a few others (23, 30, 35, 41, 42). The established sensitivity (0.2 ng/mL) is superior to a majority of the methods, except for three reports (24, 25, 30, 40). The column oven temperature was maintained at 35°C, which is considerably less than all other methods, except the work of Li *et al.* (20). This helps to maintain the column life and efficiency for more injections. A comparison of salient features of the LC–MS-MS methods developed for the

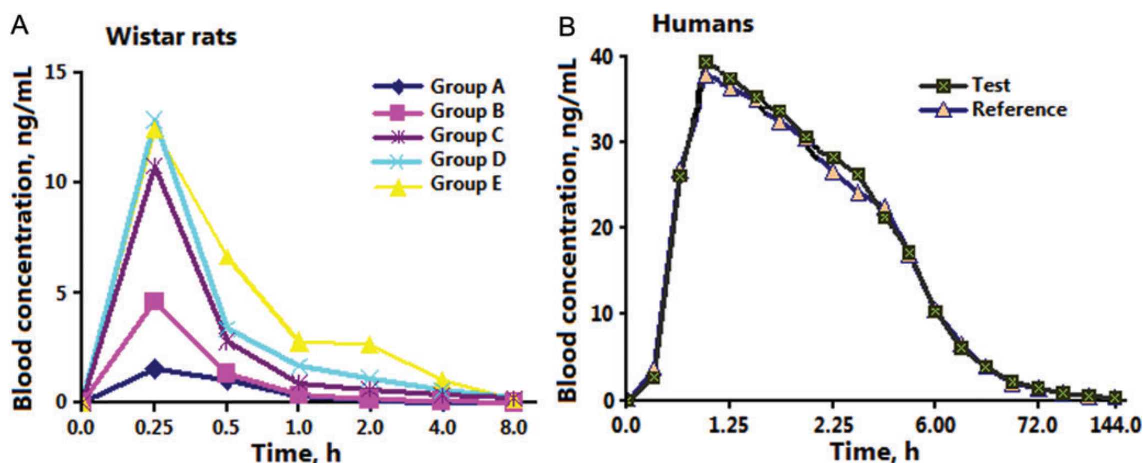

Figure 4. Mean blood concentration–time profiles of FK-506 after: oral administration of 1, 2, 3, 4 and 5 mg/kg (Groups A, B, C, D and E, respectively) of a formulation to 25 rats (A); oral administration of 5 mg (test and reference) of a capsule formulation to 20 healthy volunteers (B).

Table IV
Mean Pharmacokinetic Parameters Following Oral Administration of FK-506 (1.0–5.0 mg/kg Body Weight) to 25 Wistar Rats (Five Rats per Treatment) for a Preclinical Study

Parameter	Mean ± SD				
	Group A (1.0 mg/kg)	Group B (2.0 mg/kg)	Group C (3.0 mg/kg)	Group D (4.0 mg/kg)	Group E (5.0 mg/kg)
C_{max} (ng/mL)	1.534 ± 0.77	4.604 ± 1.65	10.788 ± 2.91	12.810 ± 4.00	12.750 ± 3.71
T_{max} (h)	0.25 ± 0.01	0.25 ± 0.01	0.25 ± 0.01	0.24 ± 0.00	0.24 ± 0.01
$t_{1/2}$ (h)	0.395 ± 0.112	0.926 ± 0.263	1.808 ± 0.423	2.322 ± 0.224	4.444 ± 0.431
AUC_{0-8} (h/ng/mL)	0.828 ± 0.550	2.043 ± 0.947	6.537 ± 1.382	8.964 ± 2.791	14.029 ± 3.163
$AUC_{0-inf.}$ (h/ng/mL)	1.391 ± 0.259	2.391 ± 0.356	8.585 ± 2.562	10.684 ± 3.051	15.435 ± 3.972

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Table V
Mean Pharmacokinetic Parameters Following Oral Administration of 5 mg Test and Reference Capsule Formulations of FK-506 to 20 Healthy Indian Subjects under Fasted Condition

Parameter	Mean ± SD	
	Test	Reference
C_{max} (ng/mL)	39.428 ± 10.655	39.205 ± 10.647
T_{max} (h)	1.167 ± 0.125	1.130 ± 0.204
$t_{1/2}$ (h)	34.501 ± 3.251	33.480 ± 3.166
AUC_{0-144} (h/ng/mL)	335.993 ± 121.964	329.386 ± 126.405
$AUC_{0-inf.}$ (h/ng/mL)	349.065 ± 128.418	330.627 ± 114.889
K_{el} (1/h)	0.020 ± 0.002	0.021 ± 0.002

determination of FK-506 in rat/human blood is given in Supplementary Table III.

Conclusions

To summarize, the UPLC–MS-MS method for the quantitation of FK-506 in rat/human blood was developed and fully validated according to FDA guidelines. The method offers significant advantages over those previously reported in terms of blood processing volume, overall analysis time and sensitivity. With dilution reliability up to 3-fold, it is possible to extend the ULOQ to 600 ng/mL. In addition, a carryover test and post-column analyte infusion experiment are also studied in the present work. The current methods have shown adequate sensitivity and selectivity for the quantification of FK-506 in rat and human blood in pre-clinical and clinical studies, respectively. Furthermore, incurred sample reanalysis results prove the reproducibility of the validated method.

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