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Liquid chromatography–negative ion electrospray tandem mass spectrometry method for the quantification of tacrolimus in human plasma and its bioanalytical applications

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Abstract

A simple, rapid, novel and sensitive liquid chromatography–tandem mass spectrometry method was developed and validated for quantification of tacrolimus (I) in human plasma, a narrow therapeutic index, potent macrolide immunosuppressive drug. The analyte and internal standard (tamsulosin (II)) were extracted by liquid–liquid extraction with *t*-butylmethylether using a Glas-Col Multi-Pulse Vortexer. The chromatographic separation was performed on reverse phase Xterra ODS column with a mobile phase of 99% methanol and 1% 10 mM ammonium acetate buffer. The deprotonate of analyte was quantitated in negative ionization by multiple reaction monitoring (MRM) with a mass spectrometer. The mass transitions *m/z* 802.5 → 560.3 and *m/z* 407.2 → 151.9 were used to measure I and II, respectively. The assay exhibited a linear dynamic range of 0.05–25 ng/ml for tacrolimus in human plasma. The lower limit of quantitation was 50 pg/ml with a relative standard deviation of less than 20%. Acceptable precision and accuracy were obtained for concentrations over the standard curve ranges. Run time of 2 min for each sample made it possible to analyze a throughput of more than 400 human plasma samples per day. The validated method has been successfully used to analyze human plasma samples for application in comparative bioavailability studies. The tacrolimus plasma concentration profile could be obtained for pharmacokinetic study. The observed maximum plasma concentration (C_{max}) of tacrolimus (5 mg oral dose) is 440 pg/ml, time to observed maximum plasma concentration (T_{max}) is 2.5 h and elimination half-life ($T_{1/2}$) is 21 h.

Keyword: Tacrolimus

1. Introduction

Tacrolimus (FK 506, Fig. 1) is a immunosuppressive agent isolated from *Streptomyces tsukubaenesis* [1]. It is a macrolide lactone with a hemi-ketal masked α,β-diketoamide and a molecular mass of 803.5 Da [2]. It is the basis of immunosuppressive drug regimens after liver and kidney transplantation and it has also been used for heart, pancreas, bone marrow, small bowel, lung transplant and for treatment of T-cell-mediated autoimmune diseases such as allergic encephalomyelitis [1,3]. The clinical management of tacrolimus is complicated by its narrow therapeutic index, intra- and inter-individually highly variable pharmacokinetics, and the lack of a reliable correlation between dose and drug exposure. Tacrolimus is mainly metabolized by cytochrome P450 3A in the liver and small intestine [4–6] and are substrates of the ATP-binding cassette transporter P-glycoprotein [7]. Several drugs commonly used after transplantation, which are cytochrome P450 3A and/or P-glycoprotein substrates, inhibitors and/or inducers, affect blood concentrations of tacrolimus with the requirement for dose adjustments [8]. Therefore, regular therapeutic drug monitoring and blood concentration guided dosing regimens have been recommended [9–13].

The results of the pharmacokinetic assessment of tacrolimus are influenced by the biological fluid analyzed, the analytical method used and the duration of study. In general, clearance and volume of distribution are higher when calculated using plasma drug concentrations than when whole blood is used. This is because drug concentrations

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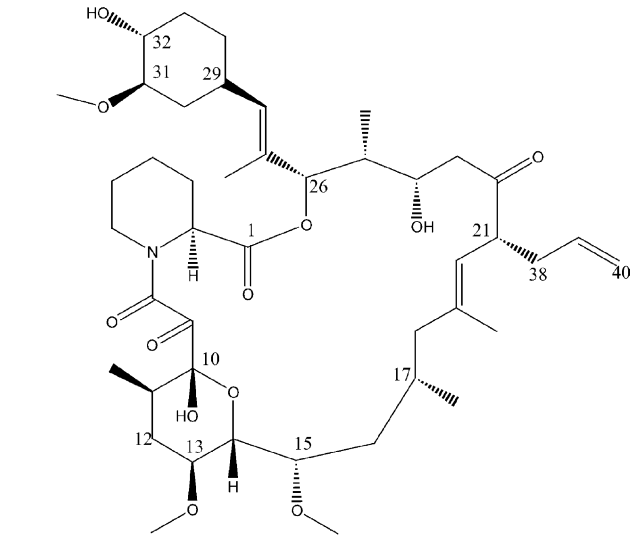
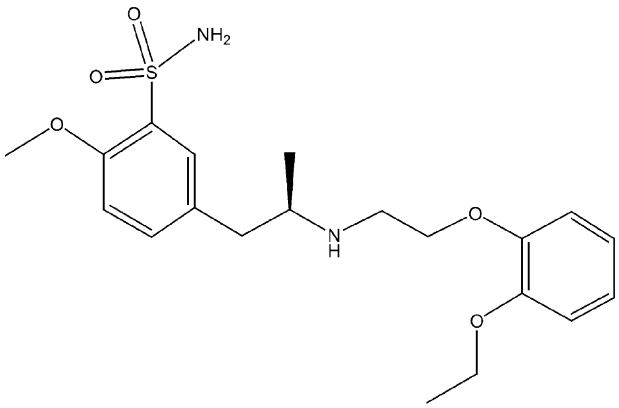


Tacrolimus (I)



Internal standard (Tamsulosin, II)

Fig. 1. Chemical structures of tacrolimus (I) and internal standard (tamsulosin (II)).

are significantly higher (mean 15 times) in the blood as a result of extensive drug binding to erythrocytes in patients with transplants [14]. Whole blood is recommended medium than plasma for assessing the pharmacokinetics of tacrolimus [3,10].

Tacrolimus is rapidly, albeit incompletely, absorbed in the gastrointestinal tract, with peak tacrolimus concentrations in whole blood or plasma (C_{max}) attained approximately 1–2 h after oral administration. Plasma protein binding may be as high as 99%, with the majority of the drug bound to α1-acid glycoprotein and albumin. Tacrolimus undergoes extensive metabolism in the liver, with less than 1% unchanged drug excreted in the urine. Tacrolimus is converted by hydroxylation and demethylation to at least 15 metabolites, with the main metabolite being 13-*O*-demethyl-tacrolimus. The mean terminal elimination half-life in adult renal or liver transplant recipients was approximately 19 and 12 h, whereas that in adult healthy volunteers was approximately 35 h [14,15].

Several analytical methods are available for therapeutic drug monitoring of cyclosporin, tacrolimus and sirolimus. Immunoassays for tacrolimus and cyclosporin are widely used in clinical practice. A survey of the literature indicates that immunoassays often lack specificity, especially for drugs which are extensively metabolized, the cross-reactivity affecting some immunoassays more than others [16–20]. For tacrolimus and cyclosporin the cross-reactivity of the metabolites causes a very significant (up to 60%) and unpredictable overestimation of the concentrations [19–22]. Assay techniques that provide specific tacrolimus concentration measurement with greater sensitivity, such as liquid chromatography–tandem mass spectrometry (LC–MS/MS), are now widely employed [23–27].

Essential to the evaluation of (1) the pharmacokinetics, (2) concentration–effect relationships, and (3) the application of therapeutic drug monitoring, during new immunosuppressive drug clinical trials, is the development of validated analytical methodology for the measurement of pharmacologically active drug and metabolites in biological fluids and tissues [28]. Even though the development of a simple, specific and sensitive assay method for measuring concentrations of tacrolimus is limited by the low absorption of the drug, low plasma and blood concentrations and the presence of metabolites and other drugs which may interfere with the determination of tacrolimus concentrations [14], we have developed and validated a simple, rapid, specific, sensitive and novel LC–MS/MS method in negative ionization mode using multiple reaction monitoring (MRM) technique for quantification of tacrolimus in human plasma for use in comparative bioavailability studies.

Tacrolimus tends to form strong adducts of sodium, potassium or ammonium ions in the electrospray source. Adduct formation is affected by slight changes in the mobile phase compositions. Therefore, robustness and ruggedness of the method is questionable. In negative ionization mode, we established higher sensitivity with acceptable precision and accuracy without the adduct formation. This method has proved to be robust and allowed the quantification of Prograf® in human plasma samples for application in comparative bioavailability studies. As of today, in the available literature, mass spectrometric methods were reported for quantification of tacrolimus, sirolimus and everolimus in whole blood with positive ionization mode using single/multiple reaction monitoring technique with adduct ion of the analyte [2,29–33]. Christians et al. [2] employed the sodium adducts, $[M + Na]^+$, of cyclosporine, tacrolimus, sirolimus and everolimus for mass spectrometric selected ion monitoring. Lensmeyer and Poquette [29] employed the sodium adducts, $[M + Na]^+$, of tacrolimus for mass spectrometric selected ion monitoring. Streit et al. [30] and Kirchner et al. [31] employed the sodium adducts, $[M + Na]^+$, of sirolimus for mass spectrometric selected ion monitoring, where as Taylor and Johnson [32] employed the ammonium adducts, $[M + NH_4]^+$, of tacrolimus and sirolimus for mass spectrometric selected reaction monitoring. Salm et al. [33] employed the ammonium adducts, $[M + NH_4]^+$, of

everolimus for mass spectrometric selected reaction monitoring. No mass spectrometric method was reported for the quantification of tacrolimus and its structurally related compounds without the adduct formation.

In recent years, a number of laboratories have reported the use of high-throughput bioanalytical procedures using LC–MS/MS [34–55]. Our method is simple, rapid, robust, specific, sensitive and novel that makes it an attractive procedure in high-throughput bioanalysis.

2. Experimental

2.1. Chemicals

The pure substance of tacrolimus was from Vimta Labs Ltd. (Hyderabad, India). Tamsulosin (internal standard (IS)) was obtained from our R&D. Chemical structures are presented in Fig. 1. Stock solutions of tacrolimus (0.5 mg/ml) and IS (0.1 mg/ml) were separately prepared in 10 ml volumetric flasks with methanol. HPLC-grade LiChrosolv methanol and *t*-butylmethylether were from Merck (Darmstadt, Germany). Ammonium acetate was from Merck (Worli, Mumbai, India). HPLC Type I water from Milli-Q system (Millipore, Bedford, MA, USA) was used. All other chemicals were of analytical grade.

2.2. LC–MS/MS apparatus and conditions

The HPLC Agilent 1100 Series (Agilent Technologies, Waldbronn, Germany) is equipped with G1312A binary pump, G1379A degasser, G1367A autosampler equipped with a G1330B thermostat, G1316A thermostatted column compartment and G1323B control module. The chromatography was on Waters Xterra ODS column (3 μm, 100 mm × 3 mm i.d.) at 30 °C temperature. The mobile phase composition was 99% methanol and 1% 10 mM ammonium acetate buffer, which was pumped at a flow rate of 0.5 ml/min.

Mass spectrometric detection was performed on an API 4000 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) using MRM. A turbo electrospray interface in negative ionization mode was used. The main working parameters of the mass spectrometer are summarized in Table 1. Data processing was performed on Analyst 1.3 software package (SCIEX).

2.3. Sample processing

A 1 ml volume of plasma sample was transferred to a 15 ml glass test tube and then 50 μl of IS solution was spiked. After vortexing for 30 s, 5 ml aliquot of extraction medium (*t*-butylmethylether) was added using dispensette organic (Brand GmbH, Postfach, Germany). The analyte and IS were extracted from plasma by vortexing for 5 min using Multi-Pulse Vortexer (Glas-Col, Terre Haute, USA). Then the sample was centrifuged using Multifuge 3S-R (Kendro Laboratory Products, Sorvall-Heraeus, Germany) for 5 min at 2000 rpm. After settled, the organic layer was quantitatively transferred to a 5 ml zymark glass tube and evaporated to dryness using TurboVap LV Evaporator (Zymark, Hopkinton, MA, USA) at 40 °C under a stream of nitrogen. Then the dried extract was reconstituted in 150 μl of diluent (water–methanol, 50:50 (v/v)) and a 10 μl aliquot was injected into chromatographic system.

Table 1
Tandem mass spectrometer main working parameters

Parameter	Value
Source temperature (°C)	350
Dwell time per transition (ms)	200
Ion source gas (Gas 1) (psi)	25
Ion source gas (Gas 2) (psi)	25
Curtain gas (psi)	14
Collision gas (psi)	5
Ion spray voltage (V)	−4400
Declustering potential (DP) (V)	−120
Entrance potential (V)	−10
Collision energy (V)	−30
Collision cell exit potential (V)	−18
Mode of analysis	Negative
Ion transition for tacrolimus, *m/z*	802.5/560.3
Ion transition for tamsulosin, *m/z*	407.2/151.9

2.4. Bioanalytical method validation

2.4.1. Calibration and control samples

Working solutions for calibration and controls were prepared from the stock solution by an adequate dilution using diluent. The IS working solution (400 ng/ml) was prepared by diluting its stock solution with diluent. Working solutions were added to drug-free plasma to obtain the tacrolimus concentration levels of 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 25 ng/ml. Seeded quality control (SQC) samples were prepared as a bulk, at concentrations of 0.05 ng/ml (LLOQ), 0.2 ng/ml (low), 11.0 ng/ml (medium) and 22.0 ng/ml (high).

2.4.2. Calibration curve

A calibration curve was constructed from a blank sample (a plasma sample processed without an IS), a zero sample (a plasma processed with IS) and nine non-zero samples covering the total range (0.05–25 ng/ml), including lower limit of quantification (LLOQ). Eight samples of each concentration were measured. Linearity was assessed by a weighted ($1/x^2$) least squares regression analysis. The calibration curve had to have a correlation coefficient (r^2) of 0.99 or better. The acceptance criterion for each back-calculated standard concentration was 15% deviation from the nominal value except LLOQ, which was set at 20%.

2.4.3. Precision and accuracy

The within-batch precision and accuracy was determined by analyzing six sets of quality control samples in a batch.

The between-batch precision and accuracy was determined by analyzing six sets of seeded quality control samples on four different batches. The quality control samples were randomized daily, processed and analyzed in position either (a) immediately following the standard curve, (b) in the middle of the batch, or (c) at the end of the batch. The acceptance criteria of within- and between-batch precision were 20% or better for LLOQ and 15% or better for the rest of concentrations and the accuracy was 100 ± 20% or better for LLOQ and 100 ± 15% or better for the rest of concentrations.

2.4.4. Recovery

Recovery of tacrolimus was evaluated by comparing the mean peak areas of six extracted low, medium and high quality control samples to mean peak areas of six neat reference solutions (unprocessed). Recovery of IS was evaluated by comparing the mean peak areas of 10 extracted quality control samples to mean peak areas of 10 neat reference solutions (unprocessed) of the same concentration.

3. Results and discussion

Electrospray MS–MS was used to analyze the compound. Negative ionization was selected to quantify the analyte because negative ion mass spectrometry gave a deprotonated molecular ion without adduct formation over positive ionization (Fig. 2). The combination of chromatographic separation by HPLC and successive mass filtrations by monitoring the transition of the deprotonated ion to product ion, provided excellent specificity for tacrolimus and internal standard. The negative ion electrospray mass spectrum of the analyte and IS gave a deprotonated molecular ions at *m*/*z* 802.5, *m*/*z* 407.2 and product ions at *m*/*z* 560.3, *m*/*z* 151.9, respectively. LC–MRM is a very powerful technique for pharmacokinetic studies since it provides sensitivity, selectivity and specificity requirements for analytical methods. Thus, the MRM technique was chosen for the assay development. The MRM state file parameters were optimized to maximize the response for the analyte.

The approach applied to the development of this method was based on the literature survey done on tacrolimus, sirolimus and everolimus, which form adducts for the quantitation by LC–MS/MS [2,29–33]. Adducts are affected by the slight changes in mobile phase compositions which in turn affect the signal intensities. Kirchner et al. [31] states addition of sodium to the mobile phase may be required to his method, if it has to be reproduced in another laboratory environment, although addition of sodium to the mobile phase did not changed signal intensities in his laboratory. So, signal intensities are affected by the presence of ion in the mobile phase concentrations. Signal intensities are also affected by the type of adduct formed. Taylor and Johnson [32] states that the resultant product ion spectra of the sodium adduct contains more fragmentation and thus give a less sensitive response for selected reaction monitoring

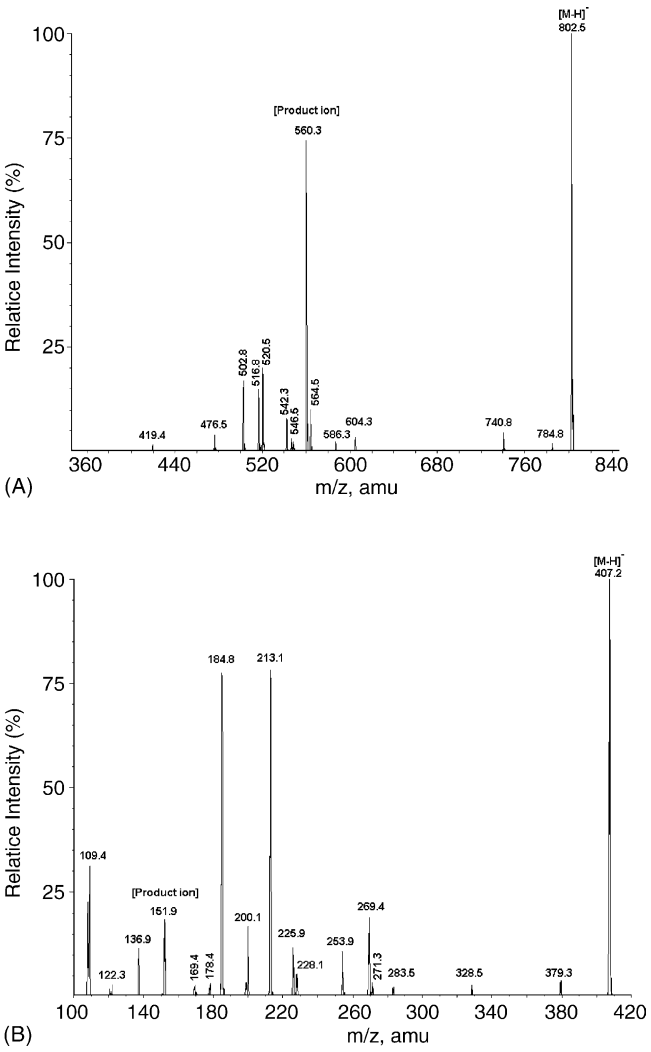


Fig. 2. Representative negative ion LC−ESI−MS−MS spectra obtained for (A) tacrolimus and (B) the internal standard. Tacrolimus and IS yielded product ions at *m*/*z* 560.3 and *m*/*z* 151.9, respectively.

than the sirolimus ammoniated species. Sodium adduct of sirolimus is more stable than the ammonium adduct and thus requires more energy to produce fragmentation. So they added ammonium acetate to the mobile phase in order to produce ammoniated species, $[M + NH_4]^+$, for mass spectrometric detection of sirolimus using selected reaction monitoring. But in case of Christians et al. [2] method, even if 2 mM ammonium acetate was added to the mobile phase to induce formation of $[M + NH_4]^+$ at the expense of other ion species, $[M + Na]^+$ still gave a significant signal. Addition of sodium ions to the mobile phase almost completely suppressed formation of other ions. So, they added sodium formate to the mobile phase for mass spectrometric detection of cyclosporine, tacrolimus, sirolimus and everolimus using selected ion monitoring. Therefore, sensitivity, robustness and ruggedness of the method are questionable. There is a need of rugged method in high-throughput bioanalysis. This method is robust, simple and rapid which makes it an attractive procedure in high-throughput bioanalysis.

3.1. Selection of mobile phase and internal standard

Different mobile phases were evaluated to improve HPLC separation and enhance sensitivity in MS. An isocratic system using a mobile phase of 99% methanol and 1% 10 mM of ammonium acetate buffer was optional for the analyte with respect to peak shape and mass spectral response. Under this condition, the retention times of both analyte and IS were approximately 1.12 min, respectively. The mobile phase used guaranteed good repeatability of retention times. The total run time for each sample was 2 min.

The use of an internal standard was required in the LC–MS/MS assay for two reasons: to compensate for loses during extraction, and to compensate for the variable detection sensitivity of the MS. The more samples we ran on the MS, the more the skimmers and the MS source were contaminated, and more the sensitivity decreased. Thus, we utilized IS (tamsulosin, Fig. 1) in this method. This molecule is a good choice because of its solubility in mobile phase and its chromatographic and extraction properties, which are similar to tacrolimus.

3.2. Calibration curves

Calibration curve was linear over the concentration range of 0.05–25 ng/ml for the analyte. The nine-point calibration curve gave acceptable results for the analyte and was used for all the calculations. The mean correlation coefficient of the weighted calibration curve generated during the validation was 0.998 for the analyte. Table 2 summarizes the calibration curve results for the analyte. The precision and accuracy for the analyte covering the concentration of 0.05–25 ng/ml ranged from 3.35 to 11.87 and 94 to 104%, respectively. The calibration curve obtained as described above was suitable for generation of acceptable data for the concentrations of the analyte in the samples during the validations.

3.3. Specificity

The specificity of the method was examined by analyzing blank human plasma extract (Fig. 3) and an extract spiked only with the internal standard (Fig. 4). As shown in Fig. 3, no significant interference in the blank plasma traces was seen from endogenous substances in drug-free human plasma at the retention time of the analyte. Fig. 4 shows the absence of interference from the internal standard to the MRM channels of the analyte. Fig. 5 depicts a representative ion-chromatogram for the LLOQ (0.05 ng/ml) of the calibration curve. Excellent sensitivity was observed for 10 μl injection volume corresponding to ca. 500 fg on-column.

Table 2
Precision and accuracy data of back-calculated concentrations of calibration samples for tacrolimus in human plasma

Nominal concentration (ng/ml)	*n*	Precision (%)	Accuracy (%)
0.05	8	9.46	94
0.10	8	8.12	104
0.20	8	3.35	96
0.50	8	6.56	98.4
1.00	8	11.87	98.7
2.00	8	5.63	101.6
5.00	8	4.06	98.3
10.00	8	6.90	102.5
25.00	8	4.58	101.7

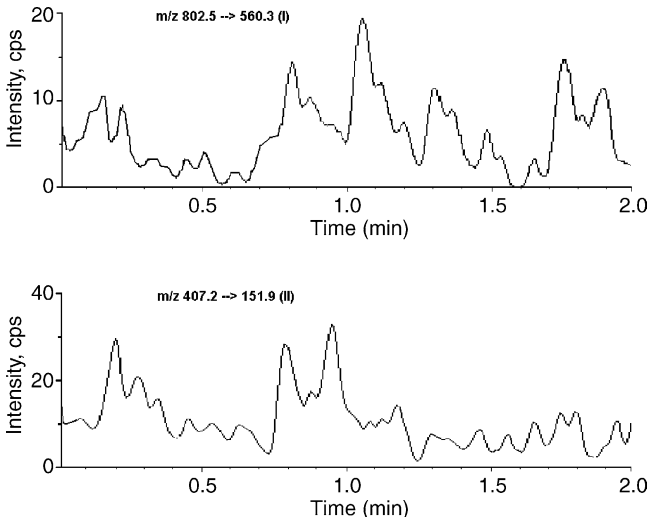


Fig. 3. LC–ESI–MS–MS ion-chromatograms resulting from the analysis of blank (drug and internal standard free) human plasma for tacrolimus (I) and IS (II).

3.4. Recovery

The extraction recovery of tacrolimus was 55.52% on average, and the dependence on concentration is negligible. The recovery of IS was 20.71% at the concentration used in

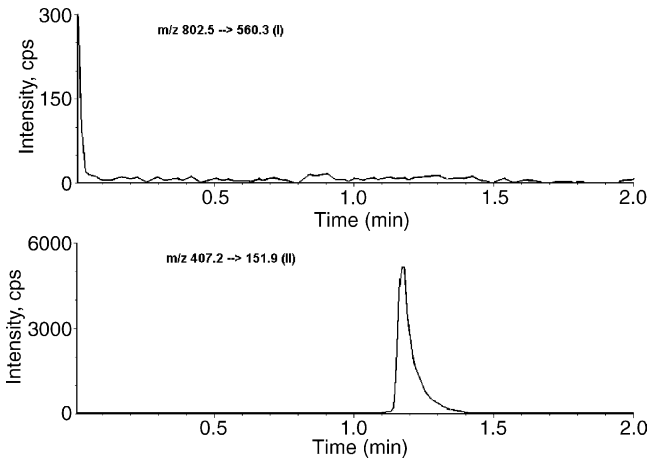


Fig. 4. LC–ESI–MS–MS ion-chromatograms resulting from the analysis of blank (drug-free spiked with IS) human plasma for tacrolimus (I) and IS (II).

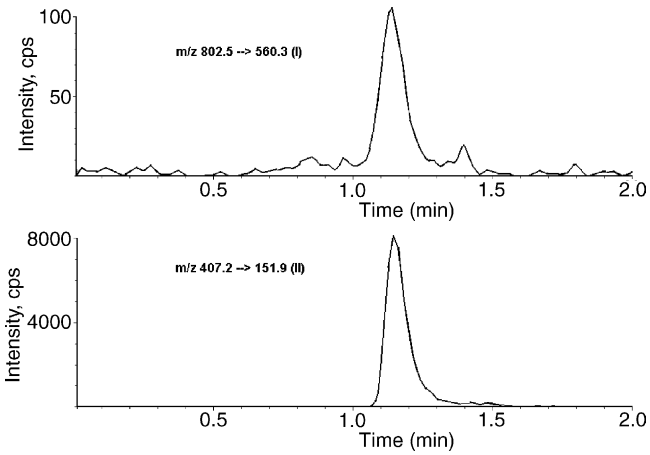


Fig. 5. Representative LC–MRM ion-chromatograms resulting from the analysis of 0.05 ng/ml (LLOQ) of tacrolimus (I) spiked with the IS (II, 20 ng per sample).

the assay (400 ng/ml). Recovery of the analyte and IS were low, but it was consistent, precise and reproducible.

3.5. Lowest concentration

The lower limit of quantitation of tacrolimus in human plasma assay was 0.05 ng/ml. Although peaks were detected at the concentration of 0.01 ng/ml with a signal-to-noise ratio above 3, the precision and accuracy did not meet the acceptance criteria (<±20%). The between-batch precision at the LLOQ—expressed as relative standard deviation (R.S.D.)—was 12.5%. The between-batch accuracy—expressed as relative error (RE)—was 103.7% (Table 3). The within-batch precision was 9.5% and the accuracy was 106.2% for tacrolimus.

3.6. Middle and upper concentrations

The middle and upper quantitation levels of tacrolimus ranged from 0.2 to 22 ng/ml in human plasma. For the between-batch experiment, the precision ranged from 6.54 to 10.63% and the accuracy ranged from 99.3 to 103.9% (Table 3). For the within-batch experiment, the precision and accuracy for the analyte met the acceptance criteria (<±15%) and precision was below 6% at all concentrations tested.

Table 3
Precision and accuracy data from between-batch experiment for tacrolimus in human plasma

Nominal concentration	*n*	Precision (%)	Accuracy (%)
LLOQ (0.05 ng/ml)	24	12.50	103.7
Low concentration (0.2 ng/ml)	24	10.63	103.9
Medium concentration (11.0 ng/ml)	24	6.54	99.3
High concentration (22.0 ng/ml)	24	6.58	100.8

3.7. Freeze−thaw stability

The freeze−thaw stability of the analyte was determined by measuring the assay precision and accuracy for the samples which underwent three freeze−thaw cycles. The stability data were used to support repeat analysis. The frozen plasma samples containing the analyte was thawed at room temperature for 2–3 h, refrozen for 12–24 h, thawed for 2–3 h, refrozen for 12–24 h, thawed and then analyzed. The results showed that the analyte was stable in human plasma through three freeze−thaw cycles. The precision ranged from 5.70 to 9.52% and the accuracy ranged from 96.7 to 102.2% (Table 4). The results demonstrated that human plasma samples could be thawed and refrozen without compromising the integrity of the samples.

3.8. Long-term storage stability

The storage time in long-term stability evaluation brackets the time between the first sample collection and the last sample analysis. The sample long-term storage stability at −72 °C was evaluated to establish acceptable storage conditions for subject samples. Aliquots of human plasma samples spiked with analyte at concentrations of 0.2, 11.0 and 22.0 ng/ml were analyzed on day 1. Then the samples from the same pools were analyzed against calibration curves from freshly prepared standards after storage at −72 °C for 30 days. The precision and accuracy for the analyte on day 30 ranged from 6.72 to 12.14 and 89.2 to 94.6%, respectively (Table 4).

3.9. Processed sample stability

Stability of the tacrolimus and its internal standard after processing in the autosampler provides advantage to determine a large number of plasma samples. Twelve sets of quality control samples were prepared as described in Section 2.3, and placed into the autosampler to +10 °C. Six sets were analyzed at once (controls) and six sets 24 h later. The results indicated that the analyte and IS were stable for at least 24 h. It took less than 10 h to run 200 samples with a sample turnover rate of 2 min per sample. This rapid assay method facilitates to analyze several hundred samples

Table 4
Stability of human plasma samples of tacrolimus

Spiked concentration (ng/ml)	*n*	Precision (%)	Accuracy (%)
Freeze−thaw stability			
0.2	6	9.52	96.7
11.0	6	5.70	102.2
22.0	6	6.15	100.8
Long-term frozen storage stability			
0.2	6	6.72	94.6
11.0	6	12.14	89.2
22.0	6	10.04	92.3

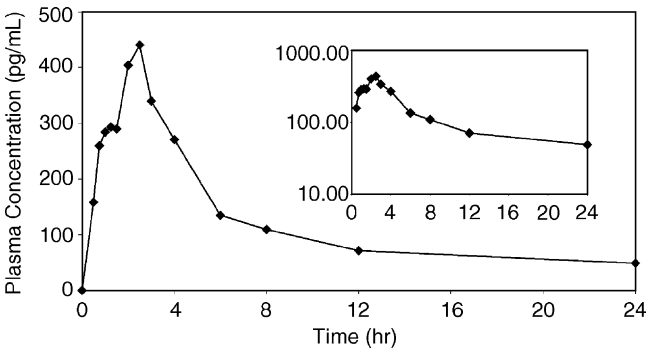


Fig. 6. Representative plasma concentration vs. time curve obtained from a subject after the oral administration of 5 mg of tacrolimus.

in 1 working day. Furthermore, tacrolimus exhibited excellent room temperature (bench top) stability for at least up to 72 h in human plasma. In addition, the stock solutions of tacrolimus and IS were also found to be stable for at least three months at 4 °C.

3.10. Application

The validated method has been successfully used to quantitate the tacrolimus concentration in the human plasma samples from a comparative bioavailability study (bioequivalence study) for a pharmaceutical research company. Since we did not own those data, an independent in-house study was carried out on a healthy subject. To demonstrate the applicability of this LC–MS/MS method to comparative bioavailability samples, it was used to quantify concentrations of tacrolimus in the plasma of the subject who received a 5 mg dose (Prograf® capsule) of tacrolimus orally. Prior to participation, this subject gave written, informed consent as approved by the Institutional Review Board. Plasma was obtained from blood samples collected prior to drug intake and up to 24 h post-dose. Plasma samples were stored frozen at −72 °C until analyzed with the procedure described above. The plasma concentration versus time curve obtained from the subject is shown in Fig. 6.

4. Conclusions

In summary, this is the first method described here for the quantification of tacrolimus from human plasma by LC–MS/MS in negative ionization mode without adducts formation using multiple reaction monitoring. The current method has shown acceptable precision and adequate sensitivity for use in bioequivalence studies of tacrolimus in healthy subjects. Furthermore, it was utilized for the analysis of hundreds of subject samples. The method described is simple, rapid, sensitive, specific and fully validated as per FDA guidelines. The cost-effectiveness, simplicity and speed of liquid–liquid extraction and sample turnover rate of 2 min. per sample make it an attractive procedure in high-throughput bioanalysis of tacrolimus. The validated method allows quantification of tacrolimus in the 0.05–25 ng/ml range.

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