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Simultaneous determination of three isomeric metabolites of tacrolimus (FK506) in human whole blood and plasma using high performance liquid chromatography–tandem mass spectrometry

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Abstract

An ammonium-adduct based liquid chromatography–tandem mass spectrometry (LC–MS/MS) method has been developed and validated for the simultaneous determination of three isomeric metabolites of tacrolimus (FK506), 13-*O*-demethylated (M1), 31-*O*-demethylated (M2) and 15-*O*-demethylated (M3) tacrolimus in human whole blood and plasma. These metabolites and the internal standards were extracted from biological matrix by methylbutyl ether (MTBE). Separation was achieved on a Genesis C_{18} column with a gradient mobile phase elution. Ammonium-adduct ions formed by a Turbo Ionspray in positive ion mode were used to detect each analyte and internal standard. The MS/MS detection was by monitoring the fragmentation of 807.5 → 772.4 (*m*/*z*) for M1, 807.5 → 754.5 (*m*/*z*) for both M2 and M3, 795.5 → 760.5 (*m*/*z*) for IS1 (FR298701) and 961.5 → 908.5 (*m*/*z*) for IS2 (FR290198) on a triple quadrupole mass spectrometer (Sciex API 3000). The retention times were approximately 4.1 min for M1, 6.8 min for M2, 6.0 min for M3, and 3.9 min for IS1 and 6.4 min for IS2, respectively. The validated dynamic range was 0.2–20 ng/ml for all three metabolites based on a sample volume of 0.25-ml. The linearity of calibration curves for M1, M2, and M3 in both matrices had a correlation coefficient of ≥0.9984. In whole blood, validation data showed intra-batch ($n = 6$) CVs of ≤5.9% and REs between −4.9 and 3.6% and inter-batch ($N = 18$) CVs of ≤4.9% and REs between −3.5 and 1.5% for all three metabolites. In human plasma, validation data showed intra-batch ($n = 6$) CVs of ≤7.3% and REs between −5.1 and 7.6% and inter-batch ($N = 18$) CVs of ≤6.6% and REs between −0.3 and 4.7% for all three metabolites. Extraction recoveries were 72% for M1, 87% for M2, 69% for M3, 79% for IS1, and 74% for IS2 from blood; and 94% for M1, 96% for M2, 98% for M3, 92% for IS1, and 93% for IS2 from plasma. All three metabolites in human blood and plasma were stable for three freeze-thaw cycles, or 24-h ambient storage, or 12 months storage at approximately −80 °C. Extracted samples were stable for at least 50 h at room temperature (RT). This method has been successfully used to analyze whole blood and plasma samples from human pharmacokinetic studies. Several key factors affecting the performance of the assay methods have also been addressed briefly.

Keywords: Tacrolimus (FK506) metabolites; Human blood and plasma; Ammonium-adduct LC–MS/MS

1. Introduction

Tacrolimus (FK506) is a well-known potent immunosuppressant that has been widely used for solid organ transplantation patients for nearly two decades [1–3]. This compound has also been introduced to treat atopic dermatitis for several years [4–6]. Some other new indications and applications of tacrolimus have been investigated as well [7,8]. There are quite a few published methods for monitoring tacrolimus levels [9–25]. These methods can be divided into two categories, immunoassays, such as enzyme-linked immunosorbant assay (ELISA), microparticle enzyme immunoassay (MEIA) [9–16] and enzyme-multiplied immunoassay technique (EMIT) [11,17–19], and chemical methods, such as LC–MS or LC–MS/MS methods [15,16,20–22]. Immunoassay methods are widely employed as routine therapeutic drug monitoring of tacrolimus for patients. However, immunoassay methodology has a relatively low sensitivity and is often lack of specificity, thus is unsuitable for pharmacokinetic (PK) studies and also hard to differentiate active metabolites from its parent drug. For tacrolimus, at least eight

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metabolites have been identified [26,27]. Among these metabolites, the major ones include 13-*O*-demethylated (M1), 31-*O*-demethylated (M2) and 15-*O*-demethylated (M3) tacrolimus, and all three of these are singly demethylated isomers (Fig. 1a). Because of their pharmacological activity and/or possible toxicity, determining the concentrations of these metabolites in clinical samples, especially for supporting PK studies, has become increasingly important. Due to their identical molecular weights and similar structures, the simultaneous and accurate determination of these isomeric metabolites in clinical samples is a very challenging task. Thus far, there is no fully validated method by which all three isomeric metabolites can be simultaneously and reliably quantified. This manuscript attempts to describe the first LC–MS/MS method for the simultaneous determination of these major tacrolimus metabolites in human whole blood and plasma.

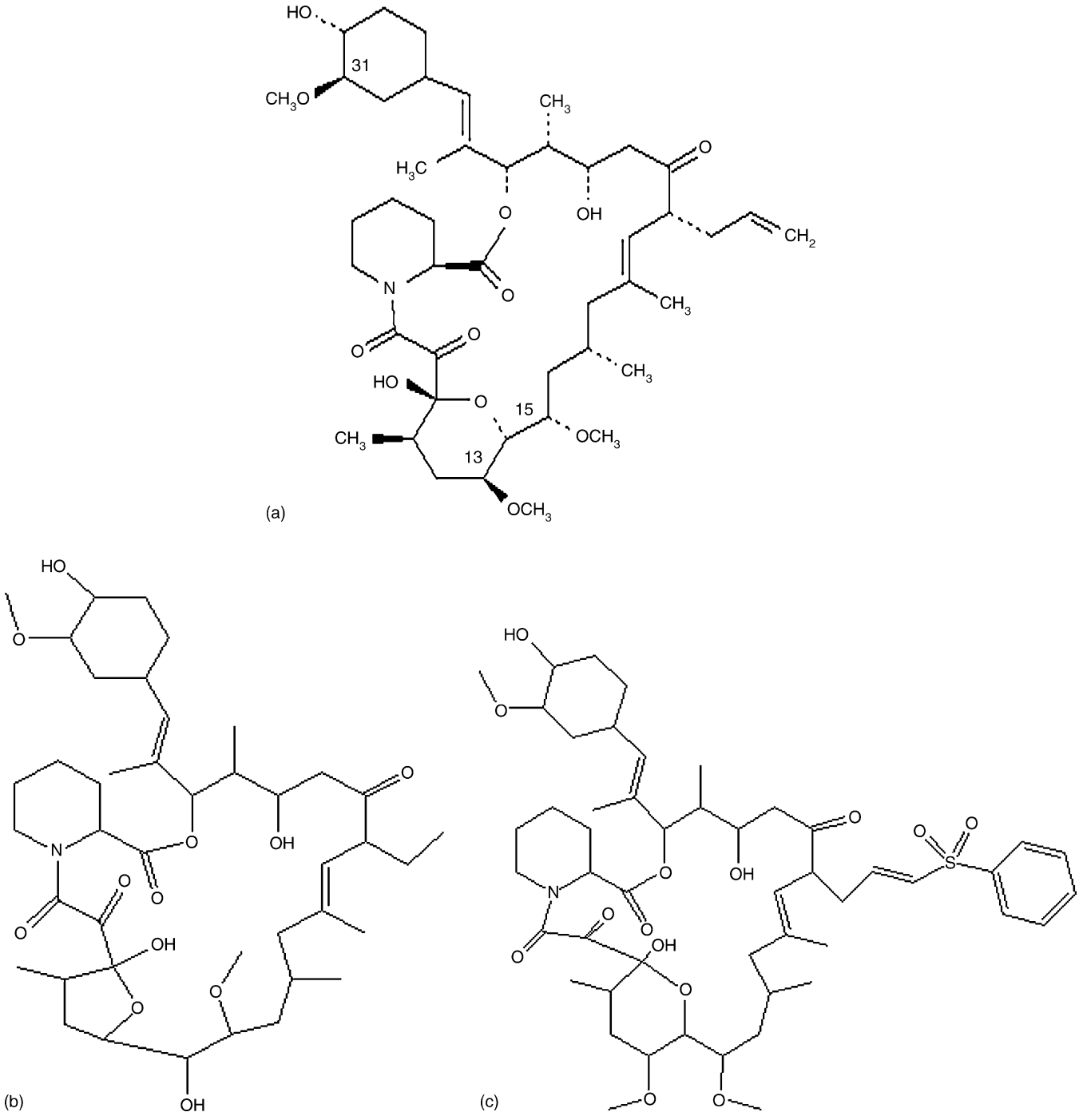

Fig. 1. Structures of tacrolimus and its major metabolites (M1, M2 and M3), and internal standards (FR298701 and FR290918): (a) tacrolimus and its major metabolites, M1 = 13-*O*-demethylated, M2 = 31-*O*-demethylated, and M3 = 15-*O*-demethylated. For each metabolite, the —OCH3 at corresponding position is replaced by a —OH group; (b) FR298701 (internal standard 1 or IS1)—the internal standard for tacrolimus-M1; (c) FR290918 (internal standard 2 or IS2)—the internal standard for tacrolimus-M2 and M3.

2. Experimental

2.1. Chemicals, materials and apparatus

All the reference materials, including three tacrolimus metabolites and two internal standards, were from Fujisawa Pharmaceuticals Co. (Osaka, Japan). M1 (13-*O*-demethylated-), M2 (31-*O*-demethylated-) and M3 (15-*O*-demethylated-) had a purity of 84.6%, 93.1%, and 80.2%, respectively. Two internal standards FR298701 (IS1, Fig. 1b) and FR290918 (IS2, Fig. 1c) were synthesized and purified by Fujisawa Pharmaceuticals Co. for research use only. The purities of FR298701 and FR290918 have been confirmed for the purpose of use as internal standards for the above metabolites. Formic acid (96%), ACS reagent, was from Alfa Aesar (Ward Hill, MA, USA). Methyl-*t*-butyl ether (MTBE), methanol, and ammonium acetate, HPLC grade, were from Fisher Scientific (Fair Lawn, New Jersey, USA). Ammonium hydroxide, certified ACS plus grade, was also from Fisher. The blank human whole blood and plasma with EDTA-K_3 anticoagulant were from Biochemed Pharmacologicals (Winchester, VA, USA). In-house high purity water was obtained from Milli-Q gradient system of Millipore (Bedford, MA, USA).

A water-bath Turbo Vap evaporator from Zymark (Hopkinton, MA, USA) was used for evaporating organic solvent from extracts. The HPLC system consisting of solvent delivery LC10ADVP, Controller SCL10ADVP and column oven CTO10ASVP was from Shimadzu (Kyoto, Japan). Sample injection was using CTC PAL autosampler from Leap Technologies (Carrboro, NC, USA). The analytical column used was a Genesis C_{18} 50 mm × 4.6 mm (5 μm) from Grace Vydac (Hesperia, CA, USA). A Turbo Ionspray ionization-triple quadrupole mass spectrometer API 3000 made by Sciex (Concord, Ont., Canada) was used for detection. Sciex *Analyst* software version 1.2 was used for data acquisition and processing.

2.2. LC–MS/MS conditions

A two-pump HPLC system was used to run a gradient mobile phase to achieve the separation. The mobile phase A was 2 mM NH_4Ac and B was 2 mM NH_4Ac in methanol with 0.1% formic acid. The time events for this gradient elution were as follows. A mobile phase of 75%B: 25%A (v/v) was initiated and maintained for 1.5 min, then B was linearly increased to 100% at 3.5 min. Between 3.5 and 8 min, the mobile phase was maintained at 100%B. From 8 to 10 min, the mobile phase was switched back to 75%B. The total flow-rate of mobile phase was 0.35 ml/min for the first 8 min, then stepped up to 0.50 ml/min for another 2 min.

A TurboIonspray ionization source operating in positive mode was used to generate ammonium-adduct ions for mass spectrometric detection. The collision energy (CE) and other parameters for the analytes and ISs were optimized by infusing each compound solution with a concentration of approximately 250 ng/ml in 70:30 methanol–water containing 2 mM NH_4Ac. The multiple-reaction mode (MRM) was used to acquire ion counts at different time points. A high voltage of 5.5 kV was applied to the spray needle. The source temperature was 450 °C and the auxiliary gas flow was 8 L/min. The settings of nebulizer gas, curtain gas, and collision gas flow at instrument were 12, 8, and 4 (arbitrary scale), respectively. All gas used in this experiment was generated from a Peak gas generator (Chicago, IL, USA). Collision energies and other optimized parameters used for each compound are presented in Table 1. In this method, both Q1 and Q3 quadrupoles were operated at unit resolution. For each injection, the total acquisition time was 10 min.

The linear regression of the peak area ratios of analyte/IS vs. concentration using a weighted $1/\text{concentration}^2$ was used to obtain calibration curves from the calibrators. The regression equations of the calibration curves were then used to calculate the blood or plasma concentration.

2.3. Standard solutions and quality control (QC) samples

The stock solutions of each analyte or IS were prepared at 100 μg/ml in methanol by vortexing approximately 1 min. Two sets of separate stock solutions were prepared for the analytes. For each IS, only one stock solution was prepared. After each analyte weighing precision of within 5% was confirmed, one set was used to make standard spiking solutions and the other used to prepare the quality control (QC) samples. Eight standard spiking solutions were prepared at concentrations of 2, 4, 10, 30, 80, 120, 160, and 200 ng/ml for each of the three metabolites. The combined IS working solution was 200 ng/ml for both ISs in methanol directly mixed and diluted from the IS stock solutions. All stock solutions and working standard solutions were stored in polypropylene vials in a −20 °C freezer. The stock solutions of M1, M2, and M3 were confirmed to be stable for at least 13 months if stored at −20 °C.

QC samples were made using the second set of stock solutions. Four levels of QC samples in both matrices were 0.200 (lower limit of quantitation, i.e. LLOQ), 0.600 (low-), 7.50 (medium-), and 15.0 (high-) ng/ml for each of the three metabo-

Table 1
Tandem mass spectrometric parameters for each compound

Compound	m.w.	Ammonium-adduct	Fragment	CE (eV)	DP (V)	FP (V)	CXP (V)	Dwell time (ms)
M1	789.5	807.5	772.4	19	71	320	12	150
M2	789.5	807.5	754.5	29	76	350	10	150
M3	789.5	807.5	754.5	27	71	360	10	150
IS1	777.5	795.5	760.5	17	71	360	6	150
IS2	943.5	961.5	908.5	35	71	360	14	150

lites. QC samples were prepared in a 50-ml pool then aliquoted into pre-labeled 2-ml polypropylene vials (approximately 0.6-ml sample per vial) and stored at −80 °C.

2.4. Sample extraction procedure

For calibration standards, an aliquot of 25.0-μl for each spiking solution was spiked into 0.250 ml of control blank matrix in a polypropylene tube. For the QC samples and study samples, an aliquot of 25-μl methanol was added to the tube to make up the volume and composition of sample matrix. Then 25.0 μl of the combined IS working solution was added to each tube and all the samples were vortex-mixed briefly. A volume of 0.50 ml 0.01 M ammonium acetate buffer (pH 7.5, adjusted by 1.5% ammonium hydroxide) was added to each sample tube. After a brief mixing, 5 ml of methyl-*t*-butyl ether was added to extract the desired compounds by vortexing 3 min and centrifugation of 5 min at 3000 rpm and 15 °C. The aqueous portion was frozen in a freezer at −80 °C and the organic portion was then transferred to a clean polypropylene tube. The organic solvent was evaporated to complete dryness at 55 °C under a stream of nitrogen at 10 psi in a Turbo-Vap water bath evaporator. The residue was reconstituted in 0.1 ml 75:25 methanol–2 mM NH_4Ac by vortexing 2 min. The resulting sample was transferred into a polypropylene HPLC injection vial followed by 10 min of centrifugation at 3000 rpm. A 10-μl aliquot of each extracted sample was injected into the LC–MS/MS system.

2.5. Method validation

The method validation experiment was designed according to the principles of the Food and Drug Administration (FDA) industry guidance [28]. For each matrix, three validation batches were processed on three separate days. Each batch included one set of calibration standards and six replicates of LLOQ, low-, medium-, and high-concentrations of QC samples. Given relatively low metabolite level, clinical samples which required dilution during the analysis were not expected, thus the validation for the effect of sample dilution with blank matrix was not intended. Freeze-thaw and room temperature (RT) stability tests for the QC samples were included in one of the validation batches. These samples were obtained by allowing QC samples to undergo three cycles of freeze-thaw or sit on bench-top for 24 h. The stability of extracted samples was established by re-injecting one of the validation batches after extracts had been at RT for at least 24 h. The 2- and 12-month frozen matrix storage stability have also been evaluated. The sample injection sequence was randomized throughout the run but always started and ended with a calibration standard in each batch.

3. Results and discussion

3.1. Mass spectrometry

Due to relatively weak proton affinity, these metabolites had low abundance of protonated molecular ions that were most often used for mass spectrometric detection in positive ionspray mode. However, these metabolites demonstrated a strong ability to bind with ammonium ion (NH_4^+) and sodium ion (Na^+) to form ammonium-adduct or sodium-adduct ions. Singly charged ammonium-adduct ions had a peak (*m*/*z*) at (m.w. + 18) and singly charged sodium-adduct ions showed a peak at (m.w. + 23). Although sodium-adducts were easily formed in the ion source, they were not suitable for MS/MS detection because of their poor fragmentation. In this method, ammonium-adduct ions were employed as precursor ions for MS/MS detection. Fig. 2 presented the MS/MS spectra of tacrolimus-metabolites and ISs. M1, M2, and M3 had the same precursor *m*/*z* peak at 807.5. M1 had several major fragment ions including 772.4, 754.5, 790.5 and 562.2, etc. with the most abundant peak at 772.4. Both M2 and M3 had several fragments with the most abundant ion at *m*/*z* of 754.5. The significant intensity of fragment ion 772.4 appeared in the MS/MS spectra of both M2 and M3. The IS1 had its ammonium-adduct peak at 795.5 with a major fragment ion of 760.5 accompanied by several other fragment ions; and IS2 had its ammonium-adduct peak at 961.5 with a dominant fragment of 908.5. Thus, MS/MS detection for above metabolites and ISs were by monitoring 807.5 → 772.4 (*m*/*z*) for M1, 807.5 → 754.5 (*m*/*z*) for both M2 and M3, 795.5 → 760.5 (*m*/*z*) for IS1 (FR298701) and 961.5 → 908.5 (*m*/*z*) for IS2 (FR290198), respectively. Collision energy, de-clustering potential (DP), focusing potential (FP), and collision cell exit potential (CXP) and dwell time optimized for each compound were summarized in Table 1. Thanks to the same precursors and fragment ions appearance in M1, M2, and M3, a baseline-separation on an analytical column is necessary to accurately determine these three metabolites. More details will be discussed in the next section.

3.2. Chromatography

M1, M2 and M3 are three chemical isomers with very limited structural differences (Fig. 1a). As shown in Fig. 2, all three metabolites had fragment ions at 754.5 and 772.4 though their relative abundance varied slightly among analytes, thus significant interference in MS or MS/MS could be expected unless an excellent chromatographic separation was achieved. Therefore, a baseline on-column separation was required for the accurate quantification of each of these three metabolites. After testing different chromatographic columns with different mobile phases, it was found that a Genesis C_{18} column (50 mm × 4.6 mm, 5 μm) with a gradient mobile phase elution was able to separate these analytes. The detail of this gradient mobile phase was described in Section 2.2. It was also noted that a relatively high column temperature (55 °C) drastically improved the separation of M2 from M3, and also minimized the peak splitting caused by tautomers. Under these separation conditions, the three metabolites were well separated from each other. The retention times were approximately 4.1 min for M1, 6.8 min for M2, 6.0 min for M3, and 3.9 min for IS1 and 6.4 min for IS2, respectively. Representative chromatograms obtained from whole blood blank, an LLOQ sample, and a clinical sample are presented in Fig. 3.

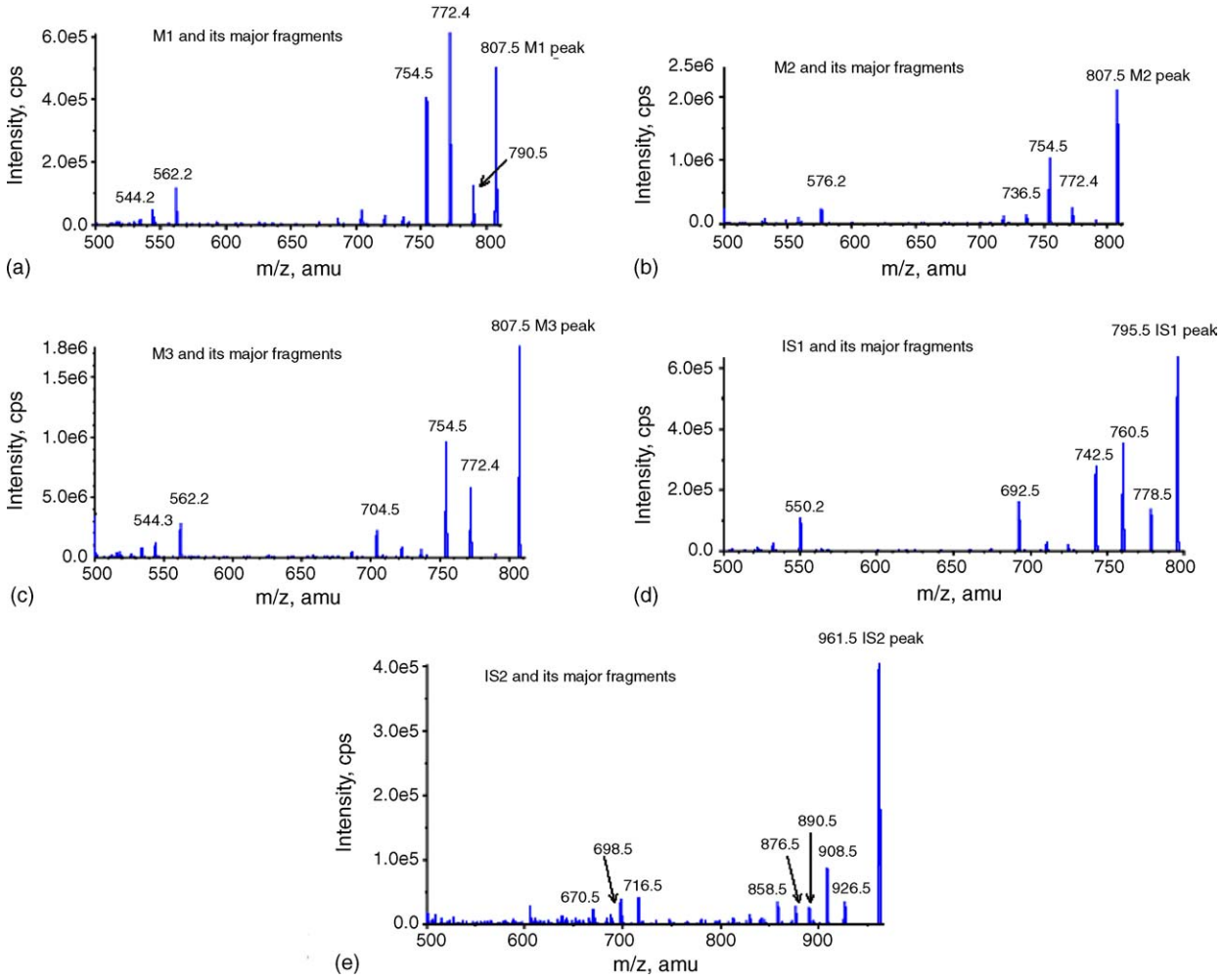

Fig. 2. (a) MS/MS spectrum of M1: M1 and its major fragments; (b) MS/MS spectrum of M2: M2 and its major fragments; (c) MS/MS spectrum of M3: M3 and its major fragments; (d) MS/MS spectrum of IS1: IS1 and its major fragments; (e) MS/MS spectrum of IS2: IS2 and its major fragments.

3.3. Selection of internal standard

The selection of an internal standard for LC–MS/MS was based on the physical and chemical similarities of the analyte and an IS candidate. A good IS should be capable of tracking extraction, on-column retention, in-source ionization and matrix effect, etc. for a target analyte. An ideal situation would have been that each analyte had a stable isotope-labeled compound as its IS. Unfortunately, this was not the case. In this case, two structural analogs were synthesized by our company in Osaka, Japan. According to their chemical structures, fragmentation (collision energy), chromatographic retention times and extraction efficiency, IS1 was very similar to M1, thus IS1 was chosen as the internal standard for M1. IS2 was eluted in between M2 and M3, and also had similar extraction recovery to that of both M2 and M3, thus IS2 was used as the internal standard for the quantitation of both M2 and M3. The validation data demonstrated that IS1 tracked M1 and IS2 tracked both M2 and M3 well. Ascomycin has been confirmed to be an excellent IS for the LC–MS/MS measurements of tacrolimus in biological samples [15,20,21,24,25]. However, it did not track tacrolimus-metabolites well because the minor structural change that occurred in these metabolites caused a drastic change in their on-column retention behavior, especially for M1. The synthesized IS1, an *O*-demethylated-ascomycin, is a more polar entity than ascomycin and has shown a better tracking for the more polar metabolite tacrolimus-M1.

3.4. Specificity and matrix effect

Six lots of blank human whole blood and six lots of blank human plasma were tested for matrix effect and the assay selectivity. For each lot of these matrices, a matrix blank (free of both analytes and ISs), control 0 (plasma blank spiked with ISs only), and control 1.00 ng/ml (spiked with 1.00 ng/ml each of M1, M2 and M3) were used to check interference and lot-to-lot matrix variation. The measured values and statistics for the spiked samples in individual lots are given in Table 2. For all tested whole blood or plasma lots, the regions of each analyte and IS were found to be free of endogenous interference. When whole blood blanks were spiked with each metabolite at 1.00 ng/ml, the measured mean, coefficient of variation (CV), and relative error (RE) were 1.04 ng/ml, 4.5%, and 4.1% for M1; 0.941 ng/ml, 10.8%, and −5.9% for M2; and 1.06 ng/ml,

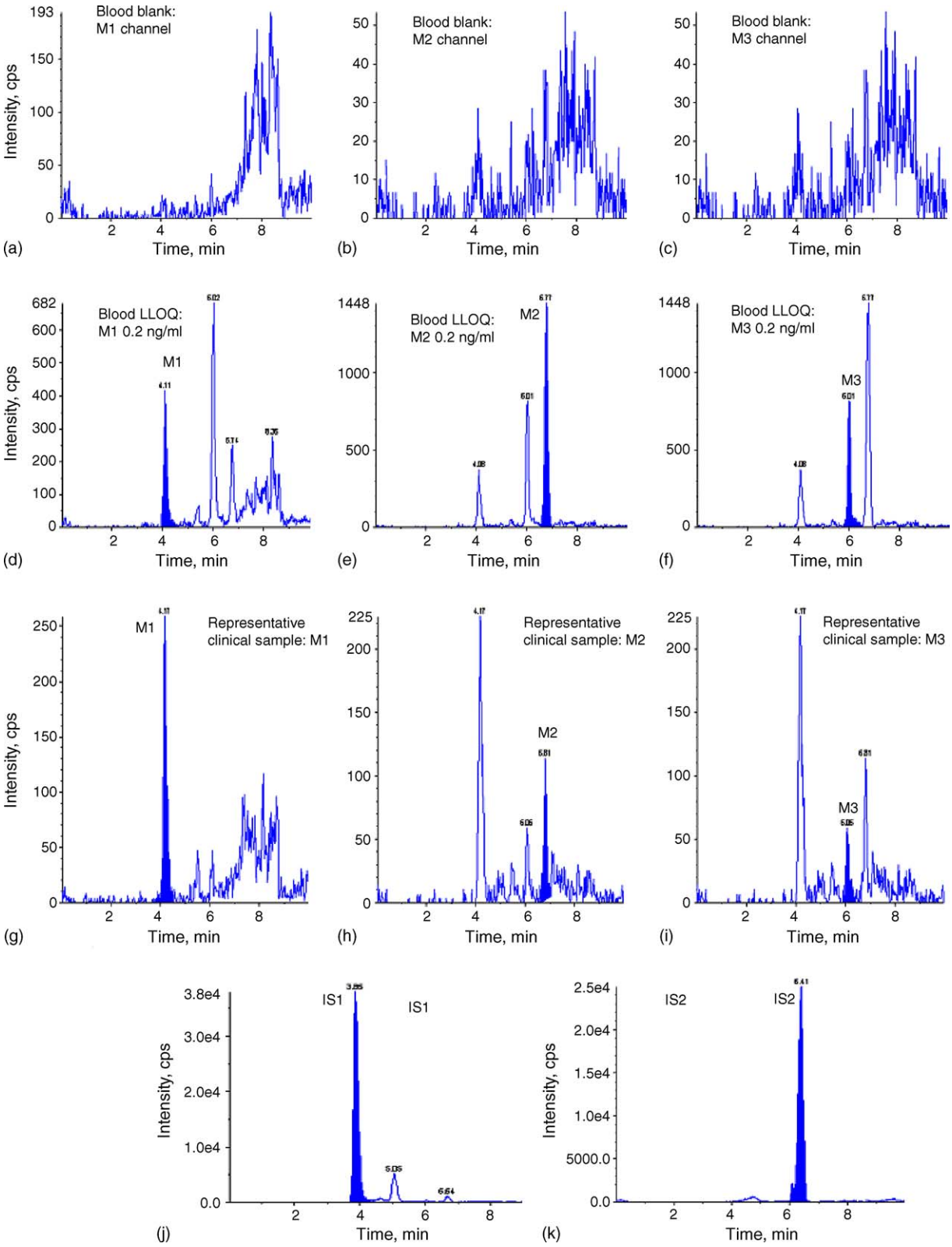

Fig. 3. Mass chromatograms obtained from a blank whole blood extract. M1 (a); M2 (b); and M3 (c). Mass chromatograms obtained from an LLOQ blood QC sample (0.2 ng/ml each of M1, M2, and M3). M1 (d); M2 (e); and M3 (f). Mass chromatograms obtained from a representative clinical human whole blood specimen, M1 (g); M2 (h); and M3 (i). For this sample all three metabolites were detectable but their actual values were lower than 0.2 ng/ml. Typical mass chromatograms of IS1 (j) and IS2 (k) obtained from extracted samples.

Table 2
Lot-to-lot variation of biological matrices

Matrix	Lot#	M1 Spiked 1.00 ng/ml	M2 Spiked 1.00 ng/ml	M3 Spiked 1.00 ng/ml
Blood	N-43829	1.02	1.05	1.05
	N-43830	1.09	1.09	1.04
	N-43832	1.08	0.884	1.05
	N-43833	1.06	0.859	1.09
	N-43837	0.963	0.868	1.03
	N-43844	1.03	0.897	1.07
	n	6	6	6
	Mean	1.04	0.941	1.06
	CV (%)	4.5	10.8	2.1
	RE (%)	4.1	−5.9	5.5
Plasma	N-29446	1.00	1.07	0.966
	N-29447	1.06	1.06	1.07
	N-30328	1.01	1.10	1.04
	N-30338	1.02	1.12	1.02
	N-30341	1.07	1.03	1.04
	N-30342	1.00	1.07	0.979
	n	6	6	6
	Mean	1.03	1.08	1.02
	CV (%)	3.0	2.9	3.9
	RE (%)	2.7	7.5	1.9

2.1%, and 5.5% for M3, respectively. For those human plasma blanks spiked with each metabolite at 1.00 ng/ml, the measured mean, CV and RE were 1.03 ng/ml, 3.0%, and 2.7% for M1; 1.02 ng/ml, 2.9%, and 7.5% for M2; and 0.966 ng/ml, 3.9%, and 1.9% for M3, respectively. These results indicated there was no significant lot-to-lot variation for both blood and plasma matrices.

3.5. Sensitivity and linearity

The calibration curve range was 0.2–20 ng/ml for M1, M2, and M3 using 0.25-ml sample for both matrices. The back-calculated concentrations (mean with CV, $n = 3$) and linear correlation coefficient for each metabolite are summarized in Table 3. In the blood method validation, the linear correlation coefficients (r^2) of calibration standards were ≥0.9995 for M1, ≥0.9985 for M2, and ≥0.9987 for M3, respectively. In the plasma validation, the linear correlation coefficients (r^2) of calibration standards were ≥0.9993 for M1, ≥0.9991 for M2, and ≥0.9984 for M3, respectively. All back-calculated values showed excellent accuracy and precision among validation batches. No single calibration standard point was dropped during the validation. An LLOQ of 0.2 ng/ml in matrices was validated for all three metabolites with acceptable precision and accuracy. The data are presented in Table 4. As shown in Fig. 3(d–f), for the whole blood LLOQ QC sample (0.2 ng/ml), M1 had a signal-to-noise (S/N) ratio of ∼30 with a peak height of 400 counts, M2 had a S/N ratio of ∼60 with a peak height of 800 counts, and M3 had a S/N ratio of ∼80 with a peak height of 1450 counts. Typically for an instrumental analysis, LOD (limit of detection) is defined to have a S/N ratio of 3. In the current LLOQ sample, S/N ratio levels for the three metabolites far exceeded that a LOD sample would require. Thus, the LLOQ of this method could be further lowered especially for M2 and M3, but this has not been experimentally confirmed yet.

3.6. Precision and accuracy

The precision and accuracy of QC samples were presented in Table 4. In whole blood, regular low-, medium- and high-QCs' validation data showed the intra-batch ($n = 6$) CVs of ≤5.9% and REs between −4.9 and 3.6% and the inter-batch ($N = 18$) CVs of ≤4.9% and REs between −3.5 and 1.5% for all three metabolites. In human plasma, regular QCs' validation data showed the intra-batch ($n = 6$) CVs of ≤7.3% and REs between −5.1 and 7.6% and the inter-batch ($N = 18$) CVs of ≤6.6% and REs between −0.3 and 4.7% for all three metabolites at three concentrations. These data showed excellent precision and accuracy of the methods across the dynamic range.

3.7. Extraction procedure and extraction recovery

A liquid–liquid extraction procedure with MTBE was developed for these metabolites and ISs. The tautomeric phenomenon of tacrolimus has been observed and two tautomers of tacrolimus were successfully isolated and characterized [29,30]. As these demethylated-tacrolimus metabolites possess a very similar molecular skeleton to that of tacrolimus, it is also very likely that they will form tautomers in solution. The equilibria among the tautomers of each metabolite were influenced by many factors, such as pH value, temperature, and solution composition, etc. Individual tautomers might have different retention times on the same analytical column, so when tautomerism was present, shoulder peak or even splitting peaks would often be observed for chromatographic analytical methods. Moreover, individual tautomers might have different extraction recoveries with the

Table 3
Back-calculated calibration standards and the linearity of calibration curves

Matrix	Analyte	STD (ng/ml)	Calibration standard level (ng/ml) ($n = 3$)							Correlation coefficient (r^2)		
			0.2	0.4	1	3	8	12	16	20	Day-1	Day-2	Day-3
Blood	M1	Back-calculated (CV%)	0.199 (1.7)	0.401 (3.9)	1.02 (0.6)	2.99 (2.0)	7.81 (0.6)	12.0 (2.1)	15.9 (0.4)	20.3 (0.5)	0.9999	0.9995	0.9997
	M2	Back-calculated (CV%)	0.203 (2.7)	0.389 (7.5)	1.00 (5.1)	2.98 (1.4)	7.90 (3.0)	12.0 (2.9)	16.0 (1.0)	20.4 (1.0)	0.9997	0.9995	0.9985
	M3	Back-calculated (CV%)	0.200 (3.5)	0.401 (6.6)	1.00 (1.3)	3.01 (1.8)	7.73 (4.3)	12.1 (0.8)	16.1 (4.4)	20.4 (1.8)	0.9995	0.9994	0.9987
Plasma	M1	Back-calculated (CV%)	0.200 (0.8)	0.400 (2.0)	1.01 (2.0)	2.90 (3.2)	7.81 (1.1)	12.0 (1.3)	16.3 (2.5)	20.6 (1.3)	0.9994	0.9993	0.9999
	M2	Back-calculated (CV%)	0.200 (0.8)	0.400 (1.4)	1.01 (1.9)	3.03 (3.7)	7.92 (1.7)	11.9 (1.7)	15.5 (3.7)	20.6 (1.0)	0.9996	0.9998	0.9991
	M3	Back-calculated (CV%)	0.199 (2.6)	0.399 (5.4)	1.02 (1.1)	3.03 (1.8)	7.94 (2.5)	11.9 (2.7)	15.1 (4.2)	20.8 (1.8)	0.9996	0.9984	0.9991

same conditions. Thus, tautomerism would certainly affect the performance of an analytical method. Our research indicated that a constant pH buffer for sample extraction was critical to have a rugged assay method for these metabolites. This type of compounds was not stable at strong basic conditions and also had a poor recovery at acidic conditions. After comparing several pH conditions, it was found that a nearly neutral pH buffer, ammonium acetate solution (0.01 M, pH 7.5, pH adjusted by 1.5%, v/v, ammonium hydroxide) was the best condition to obtain reproducible recoveries for all three metabolites and ISs. Under the above optimized conditions, average extraction recoveries were 72% for M1, 87% for M2, 69% for M3, 79% for IS1, and 74% for IS2 from whole blood; and 94% for M1, 96% for M2, 98% for M3, 92% for IS1, and 93% for IS2 from plasma. The detailed recovery data are presented in Table 5. The above results indicate that this procedure had a better extraction recovery from plasma than that from whole blood for all tested compounds. This implies that these types of compounds may have strong binding to red cells that were unable to be completely overcome by the current extraction procedure.

3.8. Short-term and long-term stability

Each stability test included six replicates of three levels of QC samples. All stability results, as well as the linear regression correlation coefficients of calibration curves generated from each stability test run for each analyte, are presented in Table 6. Whole blood QC samples undergoing three freeze-thaw cycles gave ≤5.4% CV and an accuracy of 90.0–96.8% for M1, ≤6.0% CV and an accuracy of 101–103% for M2, ≤6.7% CV and an accuracy of 102–104% for M3. Whole blood QC samples storing at ambient for 24 h gave ≤4.7% CV and an accuracy of 94.6–95.2% for M1, ≤4.6% CV and the accuracy of 93.7–96.5% for M2, ≤5.3% CV and an accuracy of 105–107% for M3. In plasma, the samples of three freeze-thaw cycles gave ≤6.0% CV and an accuracy of 97.5–103% for M1, ≤4.3% CV and an accuracy of 93–103% for M2, ≤5.8% CV and an accuracy of 92.7–104% for M3. The plasma QC samples with 24 h benchtop storage gave ≤3.8% CV and an accuracy of 89.0–92.8% for M1, ≤5.9% CV and an accuracy of 92.9–95.0% for M2, ≤4.3% CV and an accuracy of 95.2–103% for M3. The above data indicated that all three metabolites in both matrices were stable for three freeze-thaw cycles or for ambient storage of 24 h.

The tests also showed that both whole blood and plasma extracts were stable for 50 h at room temperature. A typical of within 5% CV and with ±5% RE for each analyte was observed for the sample injection 50 h after preparation. The detailed data are not presented here.

Long-term frozen storage stability was tested at 2- and 12-month after QC sample pools were prepared and stored at −80 °C. The data are also included in Table 6. The 2-month stability data of all three analytes showed an accuracy of 94.7–107% (CV ≤ 4.9%) in comparison with their theoretical values in both whole blood and plasma samples. The 12-month stability data of all three analytes had an accuracy of 94.6–106% (CV ≤ 7.4%) in both matrices. There was no indication of chemical or biological degradation or decomposition for each of three

Table 4
Intra-batch and inter-batch precision and accuracy

Matrix	Analyte	Batch #	Intra-batch ($n = 6$)												Inter-batch ($N = 18$)			
			1				2				3				over three batches			
		QC (ng/ml)	0.2	0.6	7.5	15	0.2	0.6	7.5	15	0.2	0.6	7.5	15	0.2	0.6	7.5	15
Blood		Mean (ng/ml)	0.189	0.588	7.35	15.0	0.197	0.579	7.24	14.6	0.189	0.570	7.33	14.5	0.192	0.579	7.30	14.7
	M1	CV (%)	4.3	4.5	2.1	2.7	7.6	3.6	3.6	2.2	9.3	2.2	4.1	2.7	7.2	3.6	3.2	2.8
		RE (%)	−5.5	−2.0	−2.0	0.0	−1.6	−3.4	−3.5	−2.8	−5.3	−4.9	−2.3	−3.2	−4.1	−3.5	−2.6	−2.0
		Mean (ng/ml)	0.206	0.574	7.15	14.8	0.184	0.610	7.77	15.5	0.197	0.602	7.56	15.4	0.196	0.595	7.49	15.2
	M2	CV (%)	5.1	2.1	3.9	2.6	2.7	3.0	2.9	3.1	4.8	3.0	4.1	5.3	6.4	3.7	4.9	4.2
		RE (%)	3.0	−4.3	−4.6	−1.3	−8.2	1.6	3.6	3.1	−1.5	0.3	0.7	2.7	−2.2	−0.8	−0.1	1.5
		Mean (ng/ml)	0.205	0.606	7.52	15.2	0.190	0.599	7.50	15.4	0.191	0.593	7.70	15.0	0.196	0.599	7.57	15.2
	M3	CV (%)	3.8	3.0	2.1	3.6	5.2	4.7	4.5	5.0	5.0	2.7	4.2	5.9	5.7	3.5	3.7	4.8
		RE (%)	2.7	1.1	0.2	1.4	−4.8	−0.2	0.0	2.9	−4.6	−1.2	2.6	0.0	−2.3	−0.1	0.9	1.4
Plasma		Mean (ng/ml)	0.210	0.604	7.88	15.3	0.187	0.584	7.77	15.5	0.195	0.605	7.90	15.5	0.198	0.598	7.85	15.4
	M1	CV (%)	4.0	2.8	1.6	1.8	5.9	2.4	2.6	2.9	4.6	5.4	2.5	2.2	6.7	3.9	2.3	2.3
		RE (%)	5.2	0.6	5.0	2.0	−6.3	−2.6	3.6	3.0	−2.5	0.8	5.3	3.4	−1.0	−0.3	4.7	2.7
		Mean (ng/ml)	0.202	0.601	8.02	15.6	0.204	0.616	7.91	15.9	0.196	0.580	7.60	15.0	0.201	0.599	7.84	15.5
	M2	CV (%)	5.8	1.2	3.0	2.0	4.9	2.2	3.2	4.0	5.4	3.2	2.2	3.3	5.3	3.3	3.6	4.0
		RE (%)	1.2	0.2	7.0	4.1	1.8	2.6	5.4	5.9	−2.0	−3.3	1.4	0.0	0.5	−0.2	4.5	3.3
		Mean (ng/ml)	0.210	0.597	7.57	15.5	0.187	0.629	8.07	15.1	0.208	0.57	7.57	14.9	0.201	0.598	7.74	15.1
	M3	CV (%)	5.5	5.2	3.9	3.1	7.2	7.3	6.8	5.0	3.7	2.3	2.3	2.9	7.5	6.6	5.5	3.9
		RE (%)	5.0	−0.6	1.0	3.0	−6.8	4.8	7.6	0.7	3.8	−5.1	0.9	−0.7	0.5	−0.3	3.2	0.7

Table 5
Extraction recovery (%)

	Matrix							
	Whole blood				Plasma			
	0.6 ng/ml[a]	7.5 ng/ml[a]	15 ng/ml[a]	Mean	0.6 ng/ml[a]	7.5 ng/ml[a]	15 ng/ml[a]	Mean
M1	71.7	70.6	72.1	71.5	89.4	95.9	95.9	93.8
M2	85.8	87.3	86.7	86.6	93.5	96.7	97	95.7
M3	66.9	68.4	71.8	69.0	96.6	93.9	103.5	98.0
	20 ng/ml for both ISs[b]			Mean	20 ng/ml for both ISs[b]			Mean
IS1	79.5	78.9	79.9	79.4	88.6	90.8	95.7	91.7
IS2	71.4	75.9	75.1	74.1	93.3	92.2	92.9	92.8

[a] Analyte concentration.
[b] IS concentration.

metabolites in human whole blood or plasma after a year of storage at −80 °C.

3.9. Possible interference

Specificity testing showed that there was no endogenous interference from either human blood or plasma at the elution region of each metabolite or IS. However, parent drug and other metabolite(s) may have co-elution effect or interference with these analytes. The experimental investigation indicated that tacrolimus had a retention time of 7.2 min and another major metabolite M4 (12-hydroxylated tacrolimus) had a retention time of 5.5 min under the above-established conditions for the measurement of M1, M2 and M3. These two most likely co-existing entities were completely separated from M1, M2, and M3, thus had no contribution to any of the above three metabolites and two ISs.

3.10. Key factors influencing the performance of the method

As pointed out previously, tautomerism [29,30] was an important factor that influenced the robustness and reproducibility of this assay method. A strictly controlled pH condition for extraction was able to minimize the fluctuation of tautomeric equilibrium for these tacrolimus-metabolites and secure the method although the tautomerism was unable to be eliminated. An optimized gradient mobile elution together with an appropriate column temperature was another key factor as an on-column separation was critical for the reliable quantitation of these isomeric components. One other important factor was to avoid the competition from other adduct formations, such as sodium-adduct ions. Tacrolimus has strong affinity to bind with sodium ions to form a sodium-adduct [31], and its sodium-adduct ion was used to monitor tacrolimus in blood for the therapeutic drug monitoring LC–MS methods [21,22]. In this study, authors also observed the formation of sodium-adduct ions for these metabolites and the internal standards in the process of ionization. Fig. 4 shows the competition of M1-NH_4^+ (m/z 807.5) and M1-Na^+ (m/z 812.5). Similar phenomena were observed for M2, M3 and two internal standards (data not shown). Both adduct ions had similar abundance in the presence of a mixture of 2 mM ammonium acetate and 0.2 mM sodium formate in the infusion solution. The formation of uncontrolled adduct ions would not only reduce the sensitivity of the target detec-

Table 6
Short-term and long-term stability data

Storage condition	QC (ng/ml)	In whole blood				In plasma			
		0.6	7.5	15	r^2	0.6	7.5	15	r^2
3 Freeze/thaw	M1	90.0 (5.4)	95.5 (2.3)	96.8 (2.3)	0.9997	97.5 (6.0)	103 (3.1)	103 (3.2)	0.9999
	M2	101 (6.0)	103 (1.5)	102 (3.5)	0.9985	93.0 (4.3)	103 (2.0)	99.2 (2.0)	0.9991
	M3	102 (6.7)	104 (4.3)	104 (3.7)	0.9987	92.7 (5.8)	104 (2.8)	101 (2.1)	0.9991
24-h Ambient	M1	94.6 (3.8)	95.2 (4.7)	95.1 (3.6)	0.9997	89.0 (3.8)	92.7 (3.8)	92.8 (2.9)	0.9999
	M2	94.3 (4.6)	96.5 (3.2)	93.7 (2.4)	0.9985	92.9 (5.9)	95.0 (2.4)	94.0 (1.2)	0.9991
	M3	106 (5.3)	107 (1.9)	105 (5.1)	0.9987	95.2 (4.3)	102 (1.3)	103 (2.3)	0.9991
2-month frozen (−80 °C)	M1	100 (3.6)	97.1 (2.4)	97.9 (4.3)	0.9993	102 (3.5)	101 (2.0)	98.0 (2.2)	0.9990
	M2	107 (2.9)	103 (1.7)	102 (2.5)	0.9995	94.7 (4.3)	106 (3.6)	105 (3.3)	0.9989
	M3	103 (3.5)	99.8 (2.3)	100 (2.6)	0.9996	95.0 (4.7)	103 (4.9)	103 (3.9)	0.9973
12-month frozen (−80 °C)	M1	95.2 (4.1)	94.6 (3.6)	95.4 (1.8)	0.9999	95.6 (7.5)	103 (3.4)	96.6 (2.4)	0.9993
	M2	103 (3.1)	102 (4.4)	105 (7.4)	0.9969	100 (3.4)	101 (4.1)	99.2 (4.1)	0.9975
	M3	106 (6.3)	101 (3.7)	98.3 (5.9)	0.9982	96.0 (5.7)	100 (1.4)	96.8 (6.3)	0.9970

Note: the data presented in this table are the percentage of measured value vs. theoretical value with CV in parenthesis ($n = 6$). r^2 is the linearity of the calibration curve used for this measurement.

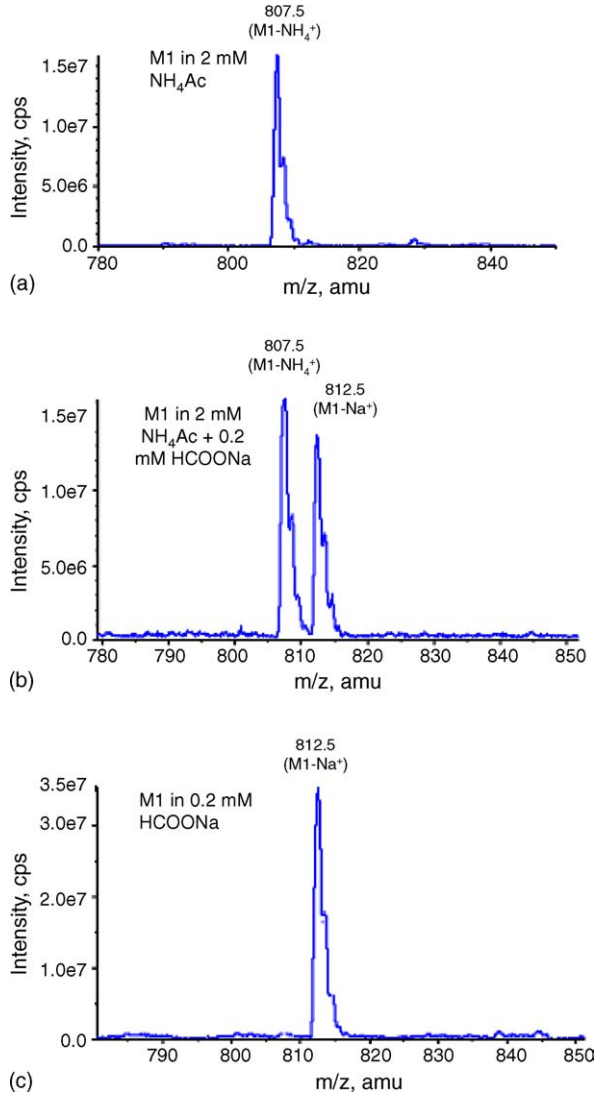

Fig. 4. Mass spectra of M1-ammonium and M1-sodium adduct ions: (a) M1 in 2 mM ammonium acetate solution; (b) M1 in a mixture of 2 mM ammonium acetate and 0.2 mM sodium formate; (c) M1 in 0.2 mM sodium formate.

tion but also affect the accuracy and precision of the method [32,33]. In this work, it was noted that the use of glassware for solutions and sample preparation would result in a highly variable intra- and inter-batch precision and accuracy. Exclusion of any potential sodium origination was a necessary measure to have a reliable ammonium-adduct based LC–MS/MS method for the measurement of tacrolimus-metabolites. Our investigation and validation experiment demonstrated that a procedure of glassware-free sample preparation and analysis was required for this assay.

3.11. Application

The above-validated methods were successfully used to analyze clinical PK whole blood and plasma samples for a Fujisawa's clinical study. In this clinical trial, all stroke patients received a single intravenous bolus dose of tacrolimus of 0.02 mg/kg. Whole blood specimens were collected at 0.083, 1, 6, 24, 48, 72, 168 and 336 h of post-dosing. Whole blood and plasma samples were analyzed for the tacrolimus levels and the individual metabolite levels separately. A substantial number of blood samples from the first 24 h showed a quantifiable M1 level but most of the samples showed M2 and M3 levels of below quantitation limits, though some of them had tiny but still detectable peaks. All plasma metabolite concentrations were much lower than those observed in the corresponding whole blood samples. Typical chromatograms obtained from a whole blood sample with detectable M1, M2 and M3 levels are presented in Fig. 3g–i.

4. Conclusions

The three most important metabolites of tacrolimus are 13-*O*-demethylated (M1), 31-*O*-demethylated (M2) and 15-*O*-demethylated (M3) tacrolimus. The simultaneous and accurate determination of these three TACROLIMUS metabolites in clinical PK samples by LC–MS/MS has been very challenging. The major reasons include (1) these metabolites possess the identical molecular weights and the same precursor ions and similar fragment ions in mass spectrometry; (2) these types of compounds tends to form tautomers in solution; and (3) these compounds have a strong affinity to form competitive metal-adduct ions. In this paper, we have discussed the influence of the above factors on the performance of the LC–MS/MS method. Through a better understanding of this mechanism, a reliable and robust LC–MS/MS method for the simultaneous and accurate measurement of three major metablolites of tacrolimus in human blood and in human plasma was successfully developed and validated. This new method has been applied to support clinical pharmacokinetic studies.

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