Development of a Simple and Rapid Method to Measure the Free Fraction of Tacrolimus in Plasma Using Ultrafiltration and LC-MS/MS

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Background: Tacrolimus is an immunosuppressant mainly used in the prophylaxis of solid organ transplant rejection. Therapeutic drug monitoring of tacrolimus is essential for avoiding toxicity related to overexposure and transplant rejection from underexposure. Previous studies suggest that unbound tacrolimus concentrations in the plasma may serve as a better predictor of tacrolimus-associated nephrotoxicity and neurotoxicity compared to tacrolimus concentration in whole blood. Monitoring the plasma concentrations of unbound tacrolimus might be of interest in preventing tacrolimus-related toxicity. Therefore, the aim was to develop a method for the measurement of total and unbound tacrolimus concentrations in plasma.

Methods: The sample preparation for the determination of the plasma concentrations of unbound tacrolimus consisted of an easy-to-use ultrafiltration method followed by solid-phase extraction. To determine the total concentration of tacrolimus in plasma, a simple method based on protein precipitation was developed. The extracts were injected into a Thermo Scientific HyPurity C18 column using gradient elution. The analytes were detected by liquid chromatography-tandem mass spectrometry with positive ionization.

Results: The method was validated over a linear range of 1.00–200 ng/L for unbound tacrolimus concentrations in plasma and 100–3200 ng/L for total plasma concentrations. The lower limit of quantification was 1.00 ng/L in ultrafiltrate and 100 ng/L in plasma. The inaccuracy and imprecision for the determination of unbound tacrolimus concentrations in ultrafiltrate and plasma showed a maximum coefficients of variation (CV) of 11.7% and a maximum bias of 3.8%.

Conclusions: A rapid and easy method based on ultrafiltration and liquid chromatography-tandem mass spectrometry was established to measure the total and unbound tacrolimus concentrations in plasma. This method can facilitate further investigations on the relationship between plasma concentrations of unbound tacrolimus and clinical outcomes in transplant recipients.

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The authors declare no conflict of interest.

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INTRODUCTION

Tacrolimus is the cornerstone of immunosuppressive therapy that can prevent rejection in solid organ transplant patients.[1] Although tacrolimus is effective, its use comes with a risk of toxicity. Neurotoxicity and nephrotoxicity are frequently observed during tacrolimus treatment, increasing the morbidity and mortality of transplant patients.[2,3] Tacrolimus has a narrow therapeutic window. Thus, the high interpatient and intrapatient variability in pharmacokinetic parameters is visualized by the high fluctuations in tacrolimus concentrations especially in the early posttransplant setting.[4] Therefore, therapeutic drug monitoring (TDM) of whole blood tacrolimus concentrations is recommended in clinical practice. Although monitoring the whole blood levels has been proven effective in preventing organ rejection,[5–9] whole blood levels show a poor association with tacrolimus-related side effects such as neurotoxicity and nephrotoxicity.[10,11] Blood binding affects the disposition of tacrolimus, and the plasma concentrations of tacrolimus were inversely correlated with the hematocrit value.[12] The average blood to plasma ratio of tacrolimus is 8 in liver and 15 in kidney and heart transplant patients, suggesting that this ratio is dependent on the nature of the organ transplanted.[13,14] The unbound fraction of tacrolimus is low (<3% of the total plasma concentration and <0.5% of the whole-blood concentration),[12,15–21] and toxicity could probably be best related to the unbound tacrolimus plasma concentrations.[22] This may be attributed to the fact that only free or unbound drug in the plasma (Fu) can migrate to tissue compartments. In previous studies on unbound tacrolimus concentrations in plasma,[18,19] the whole blood tacrolimus concentrations did not differ between organ-transplant patients who experienced tacrolimus-related toxicity and those who did not. In contrast, the unbound tacrolimus concentrations were observed to be significantly higher in patients experiencing tacrolimus-related toxicity.[19] These findings suggest that the unbound concentrations of tacrolimus correlate better with toxicity than the whole blood concentrations do. Therefore, the plasma concentrations of

unbound tacrolimus might be useful for toxicity monitoring purposes. Nevertheless, only whole blood concentrations are used for monitoring because the analysis of the unbound tacrolimus concentration in plasma is complex and is currently not available for everyday practice. Moreover, the assay for unbound concentration may be inaccurate owing to temperature-dependent distribution into the whole blood and plasma[23] and challenges associated with assay sensitivity. To further investigate the relationship between unbound tacrolimus concentration and clinical outcomes in the early posttransplant population, we aimed to develop a bioanalytical method to quantify the unbound tacrolimus concentrations in the plasma.

MATERIALS AND METHODS

Chemicals and Reagents

Tacrolimus was purchased from Sigma-Aldrich (Munich, Germany), and the internal standard (IS) tacrolimus [^{13}C,$^{2}H_2$] was purchased from Alsachim (Strasbourg, France). Water with 0.1% ammonium acetate was obtained from Sigma-Aldrich. Acetonitrile, methanol, and water were purchased from Biosolve (Valkenswaard, the Netherlands). Zinc sulfate was obtained from Merck (Darmstadt, Germany). For the isolation of unbound tacrolimus concentration in the plasma, Centrifree ultrafiltration devices from Merck Millipore (Darmstadt, Germany) were used. The OASIS HLB solid-phase extraction cartridge was obtained from Waters (Milford, CT). Newborn calf serum was obtained from Gibco-Life technologies (Paisley, Scotland, United Kingdom).

Calibrators and Quality Control Samples

For the determination of unbound tacrolimus plasma concentration, a stock solution of tacrolimus at a concentration of 500 μg/L was prepared and diluted with methanol to prepare solutions having concentrations of 0.25 μg/L and 1.25 μg/L. From these dilutions, calibrators were prepared in phosphate buffer at concentrations of 1.00, 5.00, 20.0, 50.0, 100, and 200 ng/L. The quality control (QC) samples were prepared from a second stock solution of tacrolimus. The lower limit of quantification (LLOQ), and the other controls were prepared in phosphate buffer at concentrations of 1.00, 30.0, 75.0, and 150 ng/L. The calibrators and QC solutions were diluted twice with methanol before solid-phase extraction. For the determination of total tacrolimus plasma concentration, the stock solution of tacrolimus was diluted with methanol to a concentration of 10 μg/L. From this solution, calibrators were prepared in newborn calf serum at concentrations of 100, 200, 400, 800, 1600, and 3200 ng/L. The quality control (QC) samples were prepared from a second stock solution of tacrolimus. The LLOQ, and the other controls were prepared in newborn calf serum at concentrations of 100, 500, 1500, and 3000 ng/L. For the preparation of the IS tacrolimus [^{13}C,$^{2}H_2$], a stock solution was diluted with methanol to obtain a concentration of 10 μg/L.

Sample Preparation for Unbound Tacrolimus Concentration in Plasma

A 1.5 mL aliquot of plasma was distributed over 3 Centrifree ultrafiltration devices (Merck Millipore). The filled ultrafiltration devices were centrifuged (2500*g*) for 60 minutes at 25°C. After centrifugation, the filtrates were pooled. A 500 μL aliquot of ultrafiltrate was diluted twice with methanol, and to this dilution, 50 μL IS tacrolimus [^{13}C,$^{2}H_2$] was added. The solid-phase extraction method described by Annesley and Clayton[24] for immunosuppressant drugs in whole blood was optimized and used for the determination of plasma concentration of unbound tacrolimus. Solid-phase extraction was performed with a 30 mg, 1 mL Waters OASIS HLB cartridge. The cartridge was conditioned with 1 mL of methanol followed by 1 mL of water. The sample was slowly transferred through the cartridge. The cartridge was washed twice with 1 mL of water and air-dried under reduced pressure. Tacrolimus was eluted into a clean test tube with 1 mL of acetonitrile. Thereafter, acetonitrile was evaporated under nitrogen. The residue was reconstituted in 50 μL methanol/water [50/50 (vol/vol)]. After vortexing, the volumes were inserted into glass vials containing inserts. A 25 μL aliquot of the sample was injected into the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system.

Sample Preparation for Total Plasma Concentration

To a 200 μL aliquot of plasma sample, 200 μL each of 0.1 mol/L zinc sulfate, IS tacrolimus [^{13}C,$^{2}H_2$], and methanol were added. After vortexing, the samples were centrifuged at 11,290*g* for 5 minutes. A 25 μL aliquot of the sample was injected into the LC-MS/MS system.

Instrumentation and Conditions

Tacrolimus was quantified using the Thermo Scientific (Waltham, MA) Quantiva LC-MS/MS system with an Ultimate 3000 UHPLC. The Quantiva mass spectrometer was operated in positive electrospray ionization and selected reaction monitoring mode. A method for analyzing tacrolimus in whole blood, previously described by Koster et al,[25] was optimized for this specific UHPLC system. The analytical column was a HyPurity C18 50 × 2.1 mm column with 3 μm particle size (Thermo Scientific). The autosampler temperature was set at 10°C, and the column temperature was kept at 60°C. Chromatographic separation was performed by

TABLE 1. Gradient

Time, min	A	B	C
0.00	5	65	30
0.36	5	65	30
0.37	5	20	75
1.00	5	12	83
1.10	5	0	95
1.60	5	0	95
1.61	5	65	30
2.50	5	65	30

means of a gradient with a flow rate of 500 μL/min and a total runtime of 2.5 minutes. The gradient was achieved using water with ammonium acetate (mobile phase A), water (mobile phase B), and methanol (mobile phase C). The gradient is represented in Table 1.

Analytes were detected by MS/MS via heated electro spray ionization (HESI) interface in selected reaction monitoring (SRM) mode. The parent ions, product ions, collision energy, and radio frequency (RF) lens were optimized in the authors' laboratory. For tacrolimus, the parent and product ions were set at a mass-to-charge ratio (m/z) of 821.5 and 768.5 m/z, respectively. For tacrolimus [^{13}C,$^{2}H_2$], the parent and product ions were set at a mass-to-charge ration (m/z) of 825.5 and 772.6 m/z, respectively. The collision energies for tacrolimus and tacrolimus [^{13}C,$^{2}H_2$] were 19 and 18 V, respectively. The RF lens 90 V was used for both compounds.

High-purity nitrogen was used as sheath gas and auxiliary gas, and argon was used as the collision gas. The cycle time was set at 0.3 seconds for both compounds. The optimum ion transfer tube temperature was 325°C, and the vaporizer temperature was maintained at 300°C. The ion spray voltage was set at 3500 V, and the sheath gas, auxiliary gas, and ion sweep gas pressures were set at 40, 25, and 1 Arb, respectively.

Method Validation

The validation of the analysis of unbound and total tacrolimus concentration in human plasma included the following parameters according to the FDA guidelines for bioanalytical validation.[26]

Selectivity

The selectivity of the method was assessed for potential matrix interferences. The chromatograms of 6 batches of blank human plasma samples and ultrafiltrate samples were evaluated to ensure that there are no interfering peaks at the retention time of tacrolimus and the IS tacrolimus [^{13}C,$^{2}H_2$].

Linearity, Inaccuracy, and Imprecision

For the unbound tacrolimus concentration in plasma, 7 calibration points in the range of 1.00–200 ng/L were used to determine linearity on 3 separate days using linear regression analyses. For the total plasma concentration, 7 calibration points in the range of 100–3200 ng/L were used to determine linearity on 3 separate days using linear regression analyses. The concentrations were calculated by linear regression using the calculated ratios of analyte/internal standard by area. For the determination of inaccuracy and imprecision, the QC samples were prepared and analyzed in 5-fold in 3 separate runs on 3 different days. Within-run, between-run, and overall coefficients of variation (CV) were calculated using 1-way ANOVA. The inaccuracy and imprecision were determined at the maximum tolerated bias and CV (20% for LLOQ, 15% for the other validation concentrations).

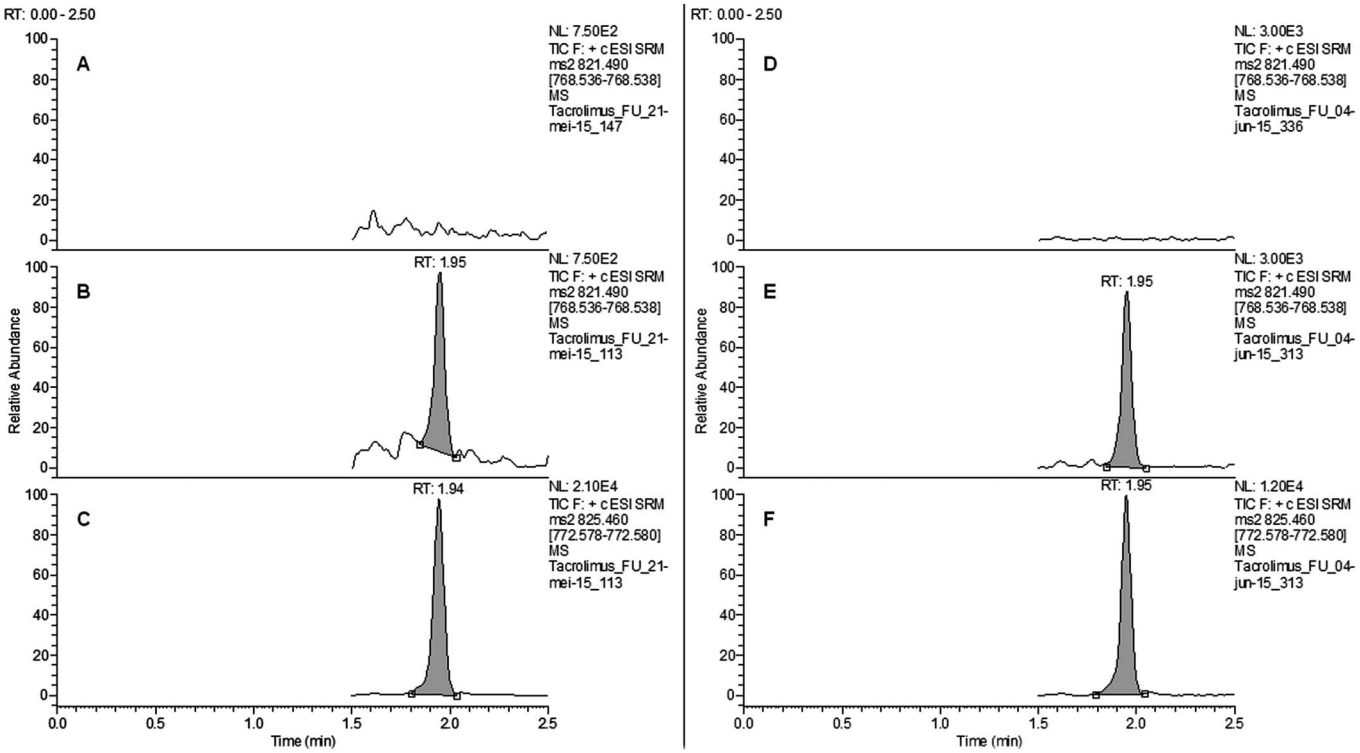

FIGURE 1. Representative chromatogram of a blank human plasma (ultrafiltrate) (A), standard at LLOQ level (ultrafiltrate) (B), internal standard (ultrafiltrate) (C), blank human plasma (D), standard at LLOQ level in plasma (E), and internal standard in plasma (F).

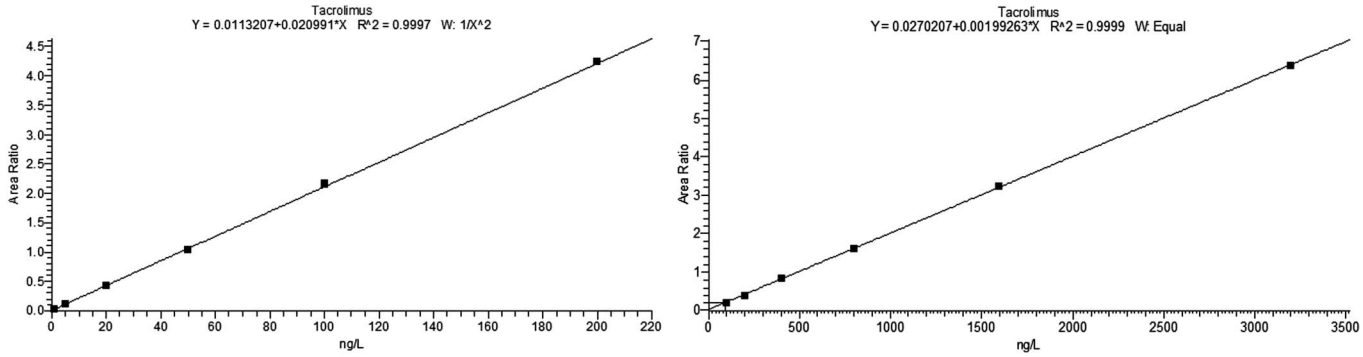

FIGURE 2. Representative calibration curves of tacrolimus in human plasma (right panel) and ultrafiltrate (left panel).

Recovery and Matrix Effects

To evaluate the extraction recovery of the solid-phase extraction, 5 replicates of blank phosphate buffer spiked with tacrolimus at a concentration of 75.0 ng/L before and after the sample preparation was compared. Six blank plasma samples spiked with tacrolimus at a concentration of 1500 ng/L before and after the sample preparation was compared. To determine the extraction recovery, the mean peak area ratio of the samples spiked before preparation was compared to the mean peak area ratio of the samples spiked after preparation. Matrix effect, expressed as matrix factors (MFs), was determined by comparing the mean area ratio response of the 5 blank plasma samples with the mean area ratio of samples prepared in methanol/water [50/50 (vol/vol)] end solution spiked at 75.0 ng/L after preparation. The matrix effect in plasma was determined by comparing the mean area ratio response of 6 blank plasma samples with the mean area ratio of samples prepared in Milli-Q water at 1500 ng/L after preparation.

Stability

The autosampler stability of tacrolimus in the ultrafiltrate was determined using a sample prepared in methanol/water [50/50 (vol/vol)] end solution. The determination of autosampler stability of tacrolimus in plasma was performed using a sample prepared in newborn calf serum. The samples were analyzed every 2 hours for a period of 20 hours at 10°C. The freeze–thaw stability at −80°C was determined using a sample prepared in newborn calf serum and analyzed in 5-fold during 3 cycles. The solutions were stable if the deviation from nominal value was less than 15%. Long-term stability was tested by storing 5 patient samples in the freezer at −80°C for 196 days.

RESULTS

Selectivity

No interference peak was detected for tacrolimus and tacrolimus [^{13}C,$^{2}H_2$] in the tested blank human plasma and ultrafiltrate. Representative chromatograms of blank human plasma, a calibrator at LLOQ level, and the internal standard are shown in Figure 1.

Linearity, Inaccuracy, and Imprecision

A weighting factor of $1/x^2$ was chosen for the determination of linearity in ultrafiltrate, and no weighting factor was chosen for the determination of linearity in plasma. The correlation coefficient (R) for both the calibration curves is shown in Figure 2.

The validation results for inaccuracy and imprecision are within the maximum tolerated bias and CV (20% for LLOQ and 15% for the other validation concentrations; Table 2).

TABLE 2. Validation Results of Unbound and Total Tacrolimus Plasma Concentrations

	Correlation Coefficient (R) (Linear Range, ng/L)	Nominal Concentration, ng/L	Mean, ng/L*	Within-Run†		Between-Run‡	
				Imprecision, CV, %	Inaccuracy, %	Imprecision, CV, %	Inaccuracy, %
Ultrafiltrate tacrolimus	0.9997 (1.00–200)	1.00	1.04 ± 0.05	9.3	2.0	1.7	3.8
		30.0	29.9 ± 0.51	2.4	−1.8	2.5	−0.3
		75.0	76.0 ± 1.52	1.3	−0.8	3.3	1.4
		150	155 ± 2.21	1.4	1.2	2.4	3.5
Plasma tacrolimus	0.9999 (100–3200)	100	101 ± 6.55	3.1	−4.5	11.3	0.7
		500	490 ± 17.2	2.0	2.7	6.0	−2.1
		1500	1461 ± 58.5	2.9	2.2	6.6	−2.6
		3000	2915 ± 120	1.2	3.6	7.3	−2.8

*Mean ± SD.
†Within-run (n = 5).
‡Between-run (n = 3).

TABLE 3. Stability Results of Unbound and Total Tacrolimus Plasma Concentrations

	Nominal Concentration, ng/L	Within-Run CV, %	Between-Run CV, %	Overall Bias, %
Ultrafiltrate tacrolimus				
F/T stability	n/a*	7.7	10.7	n/a
AS stability	5.0	2.2	n/a	10.4
Plasma tacrolimus				
F/T stability	1500	2.7	6.5	−2.7
AS stability	1500	1.1	n/a	3.2

*Nominal concentration not known.
AS, autosampler stability; F/T, stability of 3 freeze–thaw cycles.

Recovery and Matrix Effects

The extraction recovery for tacrolimus in the ultrafiltrate and plasma was 105% and 107%, respectively. The matrix effect, expressed as matrix factor, was 1.0 for tacrolimus in both ultrafiltrate and plasma. This indicated that there were no significant matrix effects for tacrolimus in the ultrafiltrate or plasma.

Stability

The results of the stability are shown in Table 3.

The unbound tacrolimus concentration in the end solution remained stable in the autosampler for 22 hours at 10°C, with a bias of 10.4% (CV 2.2%). The stability of the analyte was determined after 3 freeze–thaw cycles and evaluated by calculating the within-run and between-run coefficients of variation (CV). It is not possible to calculate the bias because we used a spiked sample; therefore, the nominal tacrolimus unbound concentration is not known. The within-run and between-run variances were both within the acceptance criteria of 15%. Five patient samples covering a concentration range of 4.75–12.2 ng/L with a median concentration of 9.72 ng/L were analyzed. Although freeze–thaw stability had no effect on the unbound tacrolimus concentration, a median increase of 37.7% with a range of 27.4%–82.5% in the unbound tacrolimus concentration was observed after the samples were stored for 196 days. The total tacrolimus concentration in the end solution was stable in the autosampler at 10°C for 22 hours, with a bias of 3.2% (CV 1.1%). The freeze–thaw stability was determined in 3 cycles and a bias of −2.7% was observed. This is within the acceptance criteria of 15%. The total plasma concentration of tacrolimus was stable in plasma stored at −80°C for at least 196 days, and a bias of less than 15% was observed.

DISCUSSION

Several techniques, such as equilibrium dialysis, ultracentrifugation, and ultrafiltration, are available for the determination of the unbound fraction of a drug. The most commonly used method for measuring the unbound fraction of a drug is equilibrium dialysis. However, depending on the properties of the compound, this method can be rather time-consuming and is not suitable for unstable compounds. As tacrolimus is a highly bound compound, it takes more time to reach equilibrium. This can cause bacterial growth and shifts in plasma pH and free fatty acid concentration. Another technique for the determination of unbound drug fraction is ultracentrifugation. The advantage of ultracentrifugation is that there are fewer issues associated with nonspecific binding to centrifugation tubes compared to that associated with binding to dialysis or ultrafiltrate membranes. However, in this method, a large amount of samples cannot be processed at once.[27] In this study, ultrafiltration was performed to separate unbound tacrolimus from the bound tacrolimus. Compared to other techniques such as ultracentrifugation and equilibrium dialysis, ultrafiltration is straightforward and easy-to-use and therefore has a higher sample throughput. Although the recovery results during ultrafiltration demonstrated no significant adsorption of the analyte to the nonspecific binding (NSB) sites, the awareness of lower and variable recoveries during this process remains warranted because it has been reported earlier.[28] Despite the risk of adsorption, ultrafiltration has many advantages.

During the first method development, the minimal sample volume required to collect a minimum of 500 μL ultrafiltrate was investigated. The ultrafiltration devices were filled with maximum 1 mL plasma, which resulted in approximately 125 μL ultrafiltrate, after 10 minutes of centrifugation. However, the unbound fraction of tacrolimus was low (<3% of the total plasma concentration and <0.5% of the whole-blood concentration),[12,15–21] and it was therefore necessary to develop a method with a low LLOQ (1 ng/L). A good signal-to-noise ratio for the tacrolimus peak in the chromatogram (Fig. 1) of LLOQ could only be achieved with minimum 500 μL of ultrafiltrate. Distributing 1.5 mL of plasma over 3 ultrafiltration tubes robustly produced at least 500 μL ultrafiltrate per sample for further sample cleanup. Thereafter, owing to the very low solubility of tacrolimus in water, and for preventing the adsorption of tacrolimus to the plastic container of the ultrafiltration device, an aliquot of 500 μL ultrafiltrate was directly diluted with methanol. In addition, newborn calf serum was used for the preparation of calibration curve and quality control samples for the determination of the total tacrolimus concentration because validation showed no interfering peaks and matrix effects. Finally, linearity in the ultrafiltrate was determined using a $1/x^2$-weighting factor, and no weighting factor was used for the determination of linearity in plasma, because for the unbound tacrolimus concentration, $1/x^2$-weighting factor showed the best fit for this large calibration range (1.00–200 ng/L).

Notably, the increase in the unbound tacrolimus concentration observed after long-term storage of the samples can be explained by the occurrence of plasma lipolysis. Lipolysis can increase the free fatty acid levels in plasma resulting in fatty acid-induced protein conformational changes. This may influence the binding of small molecules to proteins.[27] Therefore, long-term storage of plasma cannot be allowed, and the samples should be freshly ultrafiltered. For studies investigating the unbound tacrolimus concentration, "fresh" filtration shortly after the samples are drawn and thereafter long-term storage at −80°C may be recommended.

CONCLUSIONS

A fast and highly sensitive LC-MS/MS method was developed and validated for the quantitation of total and unbound plasma concentrations of tacrolimus. Compared to previously reported methods, the workflow is straightforward and easy to use, facilitating large-scale investigations on the relationship between unbound tacrolimus plasma concentrations and clinical outcomes in transplant recipients.

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