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Volumetric absorptive microsampling for the quantification of tacrolimus in capillary blood by high performance liquid chromatography-tandem mass spectrometry

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Abstract

Volumetric absorptive microsampling (VAMS) is an innovative alternative strategy to venipuncture for monitoring tacrolimus levels in transplant recipients. In this study, we aimed to validate a new high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) method for quantifying tacrolimus in blood collected by VAMS. Tacrolimus was extracted from dried blood tips in an original process involving sonication, protein precipitation and salting out. The assay was validated in accordance with EMA and IATDMCT guidelines. For clinical validation, the tacrolimus concentrations measured in liquid venous whole blood (with the reference method) were compared with those measured in capillary whole blood collected simultaneously with VAMS by a nurse. The assay was then used to monitor tacrolimus exposure in transplant recipients. The method was linear, sensitive and fast. Within-day and between-day precisions and overall bias were within $\pm 15\%$. No significant hematocrit effect was observed. The matrix effect was negligible and recovery exceeded 80% for every concentration and hematocrit levels. Tacrolimus was stable in blood collected by VAMS for 1 week at room temperature, 48 h at 60°C and 4°C and 1 month at -80°C. Clinical validation ($n=42$ paired samples) demonstrated a strong correlation between the two methods ($r = 0.97$ Pearson correlation). Bland-Altman analysis revealed that more than 90% of the differences between VAMS and liquid blood paired concentrations were within the $\pm 20\%$ acceptable range. The method had a satisfactory analytical performance and fulfilled clinical requirements. This minimally invasive VAMS-based assay appears reliable for the determination of tacrolimus levels in blood from transplanted patients.

Keywords: tacrolimus; volumetric absorptive microsampling; liquid chromatography; mass spectrometry; therapeutic drug monitoring

Abbreviations

VAMS: volumetric absorptive microsampling; TDM: therapeutic drug monitoring; HPLC-MS/MS: high performance liquid chromatography-tandem mass spectrometry; ISD: immunosuppressive drugs; AUC: area under the curve of drug concentrations; C_0 : trough concentration in whole blood; DBS: dried blood spots; MRM: multiple reaction monitoring; IS: internal standard; QC: quality controls; CV: coefficient of variation; RE: relative error; LLOQ: lower limit of quantification; EDTA: ethylenediaminetetraacetic acid; RT: room temperature; MPE: Median Prediction Error; MPPE: Median Percentage Prediction Error; RMSE: Root Median Squared Prediction Error; MAPE: Median Absolute Percentage Prediction Error

1. Introduction

Immunosuppressive drugs (ISDs) have revolutionized the management of solid organ transplant recipients, by considerably reducing the incidence of acute rejection and improving graft and patient survival [1]. Tacrolimus is the most widely prescribed ISD in solid organ transplant recipients. However, tacrolimus has a narrow therapeutic window, with low drug exposure increasing the risk of acute rejection and high exposure increasing the risk of drug toxicity (e.g., nephrotoxicity, neurotoxicity, diabetes). Therapeutic drug monitoring (TDM) is therefore essential for tacrolimus, to ensure treatment efficacy and safety through ISD dose adjustments. TDM is usually based on the determination of drug concentrations in whole blood obtained by venipuncture, which is an invasive procedure particularly because it has to be frequently repeated throughout the follow-up of the transplanted patient. Furthermore, venipuncture may prove difficult in some patients (e.g., elderly patients with a loss of vein patency, or very young patients) or should be minimized in others (e.g., frail patients, immunocompromised patients on cancer treatment). Moreover, venipuncture requires trained staff, and must be performed within institutions, such as hospitals or external biomedical analysis laboratories. As a result, for practical reasons, TDM is usually limited to a single measurement of trough concentration (C_0) in whole blood. The usual therapeutic range of tacrolimus C_0 is 3-15 ng/mL. It is assumed that C_0 is a surrogate marker of drug exposure (i.e., the area under the curve of drug concentrations (AUC)) at the time point concerned. Unfortunately, the relationship between AUC and C_0 may be weak, with coefficients of determination varying considerably between studies. That for tacrolimus, for example, has been reported to range from 0.11 to 0.94 [2,3]. In some clinical situations, the adaptation of tacrolimus dose according to AUC may therefore be a valuable alternative to C_0 determination to optimize tacrolimus treatment [4].

Microsampling approaches are therefore of increasing interest as simpler, less invasive alternative to venipuncture to perform TDM of tacrolimus. Indeed, microsamples can easily be collected by the patients themselves at home. In addition, due to the low level of invasiveness of the process, more samples can be collected than with venipuncture. Thus, microsampling could be very convenient to calculate tacrolimus AUC from concentrations measured at several sampling times within the dosing

interval. Among microsampling strategies, Dried Blood Spot (DBS) raised many hope in bioanalysis. However, hurdles have been identified for quantitative applications of DBS. (e.g., small amounts of the drug for quantification, hematocrit effect, volcano effect or volume effect that can influence the repeatability of the measurement). Most of the pre-analytical and analytical issues relating to the use of DBS should be minimized by using a volumetric adsorptive microsampling (VAMS) device to collect peripheral blood, and by performing high-performance liquid-chromatography tandem-mass spectrometry (HPLC-MS/MS) to determine drug concentrations [5,6].

The aims of this study were to develop and to validate a simple HPLC-MS/MS method for determining tacrolimus levels in blood collected with VAMS devices. The purpose of this assay is to be suitable for routine TDM, as a complementary tool to liquid venous sampling. In particular, the objectives are i) to allow home sampling to facilitate and to strengthen tacrolimus TDM based on C_0 in patient living far from the transplant centers or having intercurrent events (e.g. infection, drug-drug interaction), ii) to allows repeated sampling in hospital to monitor full AUC in specific clinical context (e.g. to assess the C_0 -AUC correlation, in case of intra-patient variability of C_0 , or in case of sides effects occurring despite C_0 in the therapeutic range). Hence, we applied the developed method to specimens obtained from transplant recipients, to demonstrate the feasibility and the suitability of the approach.

2. Materials and methods

2.1. Chemicals and materials

Tacrolimus was purchased from LGC standards (Folsheim, France). Ascomycin was purchased from Sigma-Aldrich (Saint-Quentin, France). LC/MS-grade methanol and acetonitrile were purchased from Carlo Erba Reagents (Val de Reuil, France). Ammonium sulfate, zinc sulfate heptahydrate and LC-MS-grade water were obtained from Fisher Chemicals (Waltham, USA). Calibrators and controls for lyophilized blood were purchased from Chromsystems (Gräfelfing, Germany). These samples were reconstituted in LC-MS-grade water. Blood matrix used to prepare additional quality controls and spiked samples for analytical validation experiments as well as samples used for the clinical validation were all

collected on tubes with EDTA as anticoagulant. Drug-free whole blood spiked with tacrolimus for some experiments of the analytical validation came from discarded anonymized blood samples. Spiked blood samples were gently mixed on a tumble roller for at least 2h at 37°C before proceeding to the experiment. It allowed to get binding equilibrium between blood cells that mimic *in vivo* conditions. Mitra[®] VAMS[™] devices were purchased from Neoteryx, LLC. (CA, USA).

2.2. HPLC-MS/MS equipment and instrumental conditions

2.2.1. Chromatographic conditions

A C18 Hypersil Gold column (30 x 2.1 mm, 3 µm) fitted with a guard column (10 x 2.1 mm, 3 µm) (Thermo Electron, Dreieich, Germany) was used and maintained at a constant temperature of 60 °C. The autosampler was set to 10 °C during analysis. The mobile phase consisted of a mixture of 95% acetonitrile and 5% 10 mM ammonium acetate in water. An isocratic elution mode was used, at a flow rate of 100 µL to 600 µL/min, with programming as follows: equilibration in initial conditions with a flow rate of 100 µL/min, increasing to 200 µL/min over the first minute, and then to 600 µL/min from between 1 and 1.5 minutes, before decreasing to 200 µL/min from 1.5 to 3.5 minutes. The total run time was, therefore, 2.5 minutes. Tacrolimus had a retention time of 0.6 min, and ascomycin had a retention time of 0.56 min.

2.2.2. Mass spectrometry conditions

An HPLC-Ultimate 3000 system was interfaced with a triple quadrupole mass spectrometer ENDURA MD (Thermo Fisher, San Jose, CA, USA). Positive electrospray ionization interface parameters were as follows: spray voltage 4.5 kV, sheath gas and auxiliary gas (N₂) 35 and 5 arbitrary units, respectively, capillary heater temperature 250 °C. The MS run was performed in the multiple reaction monitoring (MRM) scanning mode. The adduct [M+NH₄]⁺ transitions m/z 821.42 → m/z 768.53 (collision energy at 20 eV and tube lens value at 135 V), m/z 809.55 → m/z 756.52 (collision energy at 27 eV and tube lens value at 129 V), were monitored for tacrolimus and ascomycin, respectively. Data were acquired

and analyzed with Endura MD Tune (version 1.0.1050), LC-Quan (version 2.5.6 SP1) and TraceFinder (version 3.2.513.0) (Thermo Fisher, San Jose, CA, USA).

2.3. Preparation of standards solution, calibrators and quality control samples

A stock solution of ascomycin was prepared in acetonitrile at 500 ng/mL. This solution was stored at -80°C. The day of the analysis, a working solution was prepared at 25 ng/mL by dilution of the stock solution in water. Manufactured calibrators and controls were used. Exact whole blood concentrations of tacrolimus in these samples depended on the batch of the product. Values were provided by the manufacturer. After solubilization of the lyophilized blood in water, samples of calibrators and controls were aliquoted and stored at -20°C.

2.4. Sample preparation

Calibrators and control microsamples were prepared in blood containing tacrolimus at the desired concentration. Calibrators and controls tips were load by gently touching the blood surface with a Mitra® tip. In accordance with the instruction of the manufacturer, the tips were held in the blood samples until they turned red and then for a further two seconds. The same procedure was used to collect patient samples from a finger blood drop. Tips were dried at ambient room temperature (RT) for at least 2 h.

The Mitra® tips were then cut and dispensed into polypropylene microtubes, in which they were mixed with 100 µL LC-MS-grade water and 100 µL internal standard (IS) (ascomycin, 25 ng/mL in water) and shaken for 15 minutes (1000 rpm). The samples were sonicated, with shaking, for 10 minutes. We then added 100 µL acetonitrile, and precipitation was performed by adding 400 µL of a mixture of acetonitrile/0.05 M zinc sulfate in water (1:1 v/v). Samples were vortexed for 1 min and 100 µL ammonium sulfate 40% was added (salting out). Samples were vortexed again for 1 min and centrifuged at 16260 x g for 5 min at 8 °C. We then injected 10 µL of the supernatant into the LC-MS/MS system.

2.5. Hematocrit adjustment

For some method validation experiments, preparation of drug-free whole blood samples with known hematocrit was needed to assess impact of a range of hematocrit values on analytical performances. Hematocrits were therefore adjusted to assess the hematocrit effect, recovery and matrix effect. We adjusted hematocrit as described by Koster et al. [7]. We first measured the hematocrit of the collected drug-free blood with a flow cytometer (Sysmex, Kobe, Japan). The blood was then centrifuged for 6 min at 3000 x g and the appropriate amount of plasma was removed or added to obtain the target hematocrit. The proportion of red blood cells was checked again, after adjustment, with the automated cytometer.

2.6. Method development

We investigated several sets of sample preparation conditions to determine the best procedure for extracting tacrolimus from blood collected by VAMS. When blood samples are collected by VAMS, the first step in the extraction process is the solubilization of the dried blood and the analyte in a solvent, for extraction. First, we optimized the step of blood desorption. The most appropriate solvent for blood desorption from tips was chosen by visual inspection of the tips and was based on the assessment of precision and accuracy of tacrolimus measurements. Secondly, we also tested several mixtures of solutions for the precipitation step, including combinations of methanol, acetonitrile and aqueous zinc sulfate solution. Thirdly, we aimed to improve the sensitivity of the assay by working on the step of extract concentration.

2.7. Method validation

The analytical method was validated according to international guidelines for bioanalytical method validation from the European Medicines Agency [8]. Additional experiments were performed in accordance with guidelines from the International Association for Therapeutic Drug Monitoring and Clinical Toxicology [9].

2.7.1. Linearity

The linearity of the method was assessed by analyzing calibration samples at six concentrations, from 2.25 ng/mL to 42.9 ng/mL (the concentrations of the 6 calibrators provided by the supplier were: 2.25, 6.05, 12.1, 17.7, 24.3, 42.9 ng/mL). Calibration standards were analyzed on three different days ($n=2$ per day). Calibration curves were established by plotting the peak area ratio of the target compounds to the IS versus the nominal concentrations. A minimum of 75% of the standard calibration sample had to be in the $\pm 15\%$ range of the nominal value.

2.7.2. Accuracy and precision

Accuracy and precision were determined by replicate analysis of control samples containing known amounts of tacrolimus. Quality controls were used for four concentrations. Exact tacrolimus concentrations in blood were provided by the supplier (QC1: 2.61 ng/mL, QC2: 6.96 ng/mL, QC3: 14.6 ng/mL, QC4: 31.7 ng/mL). An additional QC was prepared by spiking drug-free blood at the concentration of 1 ng/mL to validate the LLOQ of the assay. Within-day accuracy and precision were determined by analyzing QCs in five replicates in a single validation batch. Between-day accuracy and precision were determined by repeating the analysis of five replicates over three different days. Precision was assessed by calculating the coefficient of variation (CV). Between-day precision data were analyzed by one-way ANOVA to get between-day variance according to [10]. Accuracy is expressed as the relative error (RE) or bias with respect to the theoretical value. The acceptance criteria for precision and accuracy were $\pm 15\%$ except for the LLOQ ($\pm 20\%$).

2.7.3. Carry-over

Carry-over was evaluated by injecting a high-concentration sample (highest calibration standard), immediately followed by an extracted blank sample. The peak area at the retention time of tacrolimus in the blank extract should be less than 20% that the area measured for the LLOQ sample. Carry-over should not be greater than 5% for the IS.

2.7.4. Selectivity

Selectivity was assessed with six independent sources of human blood. VAMS samples were prepared from drug-free blood and from blood spiked with tacrolimus at a concentration of 1 ng/mL (LLOQ). The lack of endogenous interference was checked by carefully examining the chromatograms of blank samples in the retention time window containing the peaks of tacrolimus or ascomycin. The blank signal should be less than 20% of the LLOQ area for tacrolimus and <5% for the internal standard (according to [9]).

2.7.5. Hematocrit effect

We investigated the effect of hematocrit on quantification accuracy, at four concentrations and three hematocrit levels. Venous blood from a single donor was used to prepare several blood samples with adjusted hematocrits (20%, 40%, 60%). These blood samples were then spiked at low (2.5 and 5 ng/mL), intermediate (15 ng/mL) or high concentration (30 ng/mL) of tacrolimus. VAMS samples from each blood sample were prepared in quintuplicate. Concentration measured in blood with a hematocrit of 40% was used as the reference for calculating the bias of the concentrations measured in blood with other hematocrits. This bias must be less than $\pm 15\%$ in order the hematocrit effect to be considered acceptable [9]. The reference hematocrit was chosen at 40% because it is the median value reported for transplanted population according to [9].

2.7.6. Influence of drying time

The use of VAMS in clinical settings for outpatients attending medical consultations will require the rapid analysis of samples in the laboratory. We therefore investigated the effect of short tip drying times on the accuracy of tacrolimus quantification. Venous blood samples from treated patients with known tacrolimus concentrations, across the therapeutic range, were used to prepare sets of VAMS tips. Concentrations were compared between tips prepared from the same source of blood dried for 30 min at 37 °C (n=8), 1 h at 37 °C (n=2) (heat chamber), 2 h at room temperature (n=2) or 24 h at room temperature (n=8). The reference drying time was 24h at 37°C.

2.7.7. Extraction recovery and matrix effect

We assessed the extraction recovery of tacrolimus from VAMS tips for three concentrations and four hematocrits. The hematocrit of human blood samples was adjusted to 25%, 30%, 40%, and 50%, and, for each hematocrit, the blood was spiked with tacrolimus at a target concentration of 5, 15 or 30 ng/mL. This resulted in 12 different blood samples from each source (4 hematocrits x 3 concentrations). The extraction procedure described above (in section 2.4) was applied to both dried blood samples on VAMS tips (10 μ L) and to 10 μ L of the same blood sample in liquid form (without tips). For a given concentration x hematocrit combination, recovery from the tips was calculated as the ratio of the area of tacrolimus peak (normalized by IS) in VAMS extract to the area of tacrolimus peak (normalized by IS) in liquid whole blood extract. Recovery rates should exceed 80 % for all samples, and the CV of recovery between blood samples at the same tacrolimus concentration but different hematocrits should be below 15%.

We also calculated process efficiency and matrix effect at several tacrolimus concentrations, with a procedure adapted from that described by Matuszewski *et al.* [11]. In a first set of experiments, relative matrix effect and process efficiency were determined in VAMS tips prepared with venous blood from six different subjects (unknown hematocrits). These blood samples were spiked with tacrolimus at two concentrations (5 and 30 ng/mL). In a second set of experiments (procedure adapted from that described by Capiou *et al.* [9]), we assessed the effect of hematocrit on matrix effect, using VAMS tips prepared with venous blood from six different subjects with hematocrit adjusted to 40% and one subject with hematocrit adjusted to 20%, 40% and 50%. In this experiment, blood samples were spiked with tacrolimus at an intermediate concentration from the usual therapeutic range (6 ng/mL). The coefficient of variation for process efficiency and matrix effect (with correction by the IS) for different blood sources or different hematocrits, should be lower than 15%.

2.7.8. Stability

We investigated the stability of tacrolimus in dried whole blood collected by VAMS, using blood from QC samples or venous blood from patients. Stability was assessed in QC samples and patients samples to cover low and high concentration levels (for each set of storage conditions). The conditions for tip storage likely to be encountered in practice were mimicked by the following storage duration and temperature conditions (tips were placed in sealed bags with desiccant): 7 days at RT, 48 h at 4 °C and 48 h at 60 °C (to mimic extreme storage conditions during transport), 1 month at RT and 1 month at -80°C. Analysis of freeze/thaw stability was not relevant to assess here since contrary to liquid whole blood, the totality of dried blood is extracted in this assay and there is no samples left to store in the freezer for further reanalysis. All samples were analyzed in triplicate, with fresh QC, and a freshly prepared calibration curve. The concentrations measured for each sample were compared to the expected concentration. Bias within $\pm 15\%$ of the nominal values was considered acceptable, and was interpreted as meaning that the tacrolimus was stable in VAMS tips under the conditions studied.

2.8. Clinical validation

The tacrolimus concentrations measured in dried capillary blood collected by fingerprick on VAMS tips was compared to those obtained for whole blood collected by venipuncture into EDTA-containing tubes (reference method). Paired blood samples were collected simultaneously from volunteer liver and kidney transplant recipients, to determine trough concentrations of tacrolimus in liquid venous whole blood and VAMS dried blood. VAMS samples were collected by nurses who beforehand received training regarding microsampling procedure. In compliance with ethical standards, all patients were informed of the purpose of this sampling and signed an information letter. The study was approved by the local ethical committee (authorization n° 20.130).

The comparison of the tacrolimus concentrations obtained with the two types of blood sample was performed on 42 paired samples. Patient hematocrit was also recorded, to assess whether difference between the two assays could be influenced by hematocrit. Pearson linear correlation was used to assess association between these two variables.

The concentrations obtained with the VAMS method and with the reference method (venous blood samples) were compared by Passing–Bablok regression.

Agreement between the two sampling methods was assessed with a Bland-Altman plot. Consistently with previous report, a clinically limit of acceptance was set as follow: relative difference between tacrolimus concentrations measured in liquid whole blood samples vs VAMS samples should be lower than $\pm 20\%$ for 80% of paired samples.

In addition, the predictive performance of the VAMS assay was assess according to guidelines and formula reported in a previous study from Veenhof *et al.* [9,12]. In brief, the following parameters were calculated: Median Prediction Error (MPE), Median Percentage Prediction Error (MPPE), Root Median Squared Prediction Error (RMSE), and Median Absolute Percentage Prediction Error (MAPE). MPPE and MAPE and should be $<15\%$.

2.9. Clinical application

Besides, the developed VAMS assay was an opportunity to estimate AUC thanks to repeated samples collections in the context of routine therapeutic drug monitoring in clinical practice. Indeed, because of low invasiveness of microsampling, patients and clinicians were keen on accepting many sample collections within a day to allow pharmacologist to accurately estimate tacrolimus exposure. We present AUC results from 10 adults kidney transplant recipients treated once daily with tacrolimus, for whom pharmacokinetic profile and individual AUC/ C_0 ratio evaluations could help to tailor tacrolimus dosage. Blood samples were collected with a VAMS device by trained nurses in patients admitted to the day hospital. All tips were visually inspected by the lab analyst before extraction to check the quality of sampling. Under-filled tips were discarded. Every patient was informed of the purpose of these sampling by clinicians. This part of the study was also approved by the local ethical committee (authorization n° 20.130). In practice, an AUC was determined for a patient upon request of the clinician, for instance in cases of adverse effects despite C_0 lying within the therapeutic range, when a drug-drug interaction was expected or when an atypical pharmacokinetic profile was suspected. Sampling times were scheduled within tacrolimus dosing interval, from morning trough (T_0) to 12h after the drug intake (usual sampling

times were: pre-dose, and 15min, 30min, 1h, 2h, 3h, 4h, 6h, 8h, 12h post-drug intake). Non-compartmental pharmacokinetic analysis (PK Solver®) was performed to estimate the AUC (linear trapezoidal method). AUC/C₀ ratios were calculated using morning trough concentration and values were interpreted according to ranges reported in Brunet et al. article [4].

3. Results

3.1. Method development

First, to optimize the step of tacrolimus desorption from dried blood, we tested sample desorption under agitation (vortex) in organic solvents, such as acetonitrile and methanol. We assumed these solvents were suitable to solubilize lipophilic drug such as tacrolimus (LogP of about 3). However, with these solvent, tips remained red meaning that blood desorption from tips was not good enough. Consistently, we observed that tacrolimus recovery from tips was highly variable when organic solvents were used for tips desorption. Better results were obtained when desorption was performed in 100% deionized water. Indeed, with an aqueous solution, the blood was seen to be completely solubilized (the tips went from red to colorless) and high and repeatable extraction recoveries were achieved for tacrolimus as well as satisfying accuracy and precision (n=3 replicate / QC concentration levels, data not shown). Thus, tips desorption process consisted in vortexing tips in water followed by a step of sonication to maximized effectiveness of desorption. Secondly, we optimized volumes and compositions of the solution used for the extraction of analytes by protein precipitation. To be able to achieve the expected LLOQ (1 ng/mL), a compromise had to be found, to achieve the cleanest possible extract with the lowest dilution of sample. The following mixtures were tested for precipitation: 100% methanol, 100% acetonitrile, 50% acetonitrile/50% 0.05 M zinc sulfate in water and 25% MeOH/25% 0.05 M zinc sulfate in water/50% acetonitrile. Various volumes, from 150 µL to 500 µL, were tested. Finally, we found that a precipitant solution including a mixture of organic solvent and aqueous zinc sulfate solution was required to obtain a limp extract. In addition, a minimum of 500 µL precipitant was needed in order the mixture was strong enough to precipitate all the extract (below this volume, clogging of the extract occurred in the HPLC system). Thirdly, we had to add a step of extract concentration to the process to

compensate for the dilution of the extract by precipitant solution. We found that salting out with ammonium sulfate was a very efficient and fast option to cope with this issue. It has the advantage to be less cumbersome than liquid-liquid extraction. This technique only works if the analyte is solubilized in a mixture of water and an organic solvent of low polarity which was the case of our extract composition. The analyte can then be concentrated in the organic supernatant, after the addition of the salt. Interestingly, we found that salting out was not compatible with precipitant solutions including methanol (the solvent remained miscible with water). At the end of this preliminary work of method development, we ultimately determined that the best precipitant solution was a mixture of 50% acetonitrile/50% 0.05 M zinc sulfate in water. The use of zinc sulfate increased the precipitation power of the solution, counterbalancing the high proportion of water in the mixture. After salting out in ammonium sulfate, tacrolimus was concentrated in 200 μ L of organic (acetonitrile) upper-layer, ready for injection into the LC-MS/MS system without further time-consuming step of evaporation and resolubilization.

3.2. Analytical validation

3.2.1. Linearity

The method was linear over the calibration range. Heteroscedasticity was checked. The best fit was obtained with a weighing factor of $1/x$. The back-calculated concentrations of the calibration standards satisfied the acceptance criteria ($\pm 15\%$) and the coefficient of correlation was > 0.99 for all calibration curves (Table S1).

3.2.2. Accuracy and precision

Within- and between-day precision and accuracy were determined at four concentrations (table 1). The acceptance criteria were satisfied, because bias and CV did not exceed 15% for any of the QCs .

3.2.3. Carry-over

No carry-over was observed in the validation experiments, as the signal for a blank sample injected after the highest calibration concentration of tacrolimus was 11.9% that obtained for the LLOQ sample. No significant carry-over was observed for ascomycin (signal ratio = 0.4%)

3.2.4. Selectivity

We observed no interference at the retention times of tacrolimus and ascomycin in blank samples extracted from six sources of human bloods. The mean (range) ratio of area of the background noise at the retention time of tacrolimus / area of the peak of analyte in the LLOQ was 3.6% (1.3-5.8%) for tacrolimus and 0.2% (0.1-0.3%) for ascomycin.

3.2.5. Hematocrit effect

The influence of hematocrit on tacrolimus concentrations (at 4 concentration levels) is reported in table 2. No significant hematocrit effect was observed relative to the standard hematocrit of 40% (relative differences <15%) in a large range of concentration (2.5 to 30 ng/mL) and a large range of hematocrit (20% to 60%)

3.2.6. Influence of drying time

Consistent tacrolimus concentrations were obtained for VAMS samples dried in the four different sets of conditions (Figure 1). Relative differences compared to concentration measured in tips dried for 24h at RT were below $\pm 15\%$. Consequently, a short drying time of blood (30 min at 37°C or 2h at RT) should provide similar results than in blood dried longer (24h at RT).

3.2.7. Extraction recovery and matrix effect

The recovery results of VAMS samples and liquid venous blood samples are reported in table 3. At each concentration and hematocrit, recovery exceeded 80% and was not affected by hematocrit, because the CV was below 10% at each concentration.

Process efficiency and matrix effect satisfied the acceptance criteria, as the coefficient of variation remained below 15% for all concentrations and hematocrits (table 4).

3.2.8. Stability

Tacrolimus was stable in VAMS tips stored for 7 days at room temperature, 48 h at 4 °C and 48 h at 60 °C. This stability indicates that VAMS is a suitable method for blood sampling at home and transport by regular mail. For long-term storage in the laboratory in case of delayed analysis, samples should not be stored at RT, but at -80 °C, as a loss of more than 20% was observed for VAMS blood analyzed after one month of storage at RT, whereas the samples remained stable at -80 °C. The stability results are reported in table 5.

3.3. Clinical validation

Concordant results were obtained when tacrolimus determinations in liquid whole blood samples collected by venipuncture were compared with determinations on blood collected simultaneously by VAMS. Passing–Bablok regression analysis is shown in figure 2 (slope =0.98 [0.91;1.11], intercept = -0.2 [-0.99;0.18]). In addition, a Bland-Altman plot showed a minor mean negative systematic bias of -0.4 ng/mL (or -5.4%) (95% CI -0.08 to -0.92) for VAMS concentrations compared to liquid whole blood concentration, which is not clinically significant (figure 3). We observed that 90.5% of the paired concentrations (liquid whole blood-VAMS) were within the limits of acceptance (relative difference of $\pm 20\%$). It fulfilled the required threshold of at least 80%. Overall, the predictive performances of VAMS assay were satisfying with a MPE of -0.33 ng/mL, a MMPE of -4.1%, a RMSE of 0.57 ng/mL and a MAPE of 7.4%.

Hematocrit of the cohort ranged from 25% to 50%. No association was found between hematocrit and absolute differences between concentrations measured in liquid whole blood or in VAMS blood samples (Pearson-test : $r=0.08$ $p\text{-value}=0.6$).

3.4. Clinical application

Tacrolimus exposure was successfully assessed in patients treated once daily with tacrolimus (Advagraf® or Envarsus®). Demographics of these patients are presented in table S2. Results of the pharmacokinetics analysis are presented in table 6. We observed that all C_0 concentrations were within the usual therapeutic range (3-15 ng/mL). However the range of AUC/ C_0 ratios was large (30.3 to 66). It suggests that for some patients, tacrolimus exposure is not well reflected by trough concentration. Mean T_{max} were consistent with values expected for both formulations of once-daily tacrolimus.

4. Discussion

The determination of tacrolimus concentrations in dried blood collected with microsampling devices is of great interest as a means of improving the follow-up of transplant recipients. The development and validation of a bioanalytical LC-MS/MS method for quantifying this cornerstone immunosuppressant in blood collected by VAMS is therefore of considerable pertinence, although several analytical challenges remain to be overcome. Many methods for the determination of tacrolimus concentrations in dried blood spots collected on paper cards have been published [13], but very few strategies based on volumetric microsampling have been described [14–18].

The method reported here is accurate, precise and sensitive. The assay was linear over a large calibration. However, dilution integrity remained to be validated in order to deal with quantification of rare higher tacrolimus concentration that could be observed in some patient (for instance at T_{max}). We used a fast extraction process based on protein precipitation with minimal centrifugation times and with no evaporation to dryness, contrasting with the more time-consuming processes reported in previous studies [16,18]. In our method, salting out with an ammonium salt was used to obtain high recovery rates and high sensitivity (low LLOQ) despite the small volume of blood collected (10 μ L). Our method is also the only VAMS tacrolimus assay to date to make use of a precipitation solution without methanol. Koster *et al.* hypothesized that methanol would increase analyte solubility and recovery [16]. However, we found that the use of an extraction solution consisting of a water-methanol mixture resulted in a cloudy supernatant, and imprecise and inaccurate results. We found that an extraction mixture consisting

of a water-acetonitrile and zinc sulfate mixture, combined with our salting-out step, resulted in better tacrolimus extraction rates. Another feature of our method is the use of ascomycin as an IS. Even though we acknowledge that deuterated IS is usually a preferable option for method with mass spectrometry detection, results of our method validation were satisfying enough to consider that ascomycin was an acceptable IS. Ascomycin was eluted almost at the same retention time than tacrolimus and this IS appeared to compensate matrix effect likely because of similarity in chemical structures of both compounds.

The main expected issue with dry blood microsampling strategy is the hematocrit effect. It should be greatly reduced by the use of volumetric blood collection methods, such as the VAMS method used here. We showed that tacrolimus measurement was not influenced by hematocrit for concentrations covering the therapeutic range and at hematocrits ranging from 20% to 60%. Most patients are expected to have hematocrit within this window but for patients with even more extreme hematocrit value, the method should not be used. We also reported that recovery was not influenced by hematocrit. As explained by Velghe *et al.* it is a crucial point that could explain why in some case a hematocrit effect still occurs despite the use of volumetric microsampling to cope with it [6]. The authors underlined that the extraction procedure of the analyte from VAMS may be a determinant factor to get reproducible recoveries. For instance, Veenhof *et al.* found that the agreement between tacrolimus concentrations in liquid whole blood and in DBS was better than the agreement between liquid whole blood and VAMS, showing extraction recovery can still be an issue for VAMS [12]. This is in accordance with our observations during the optimization of our extraction procedure since we had to try several solvent mixtures and to mix and sonicate samples a sufficient time to get satisfying results.

One challenge in the use of dried microsamples in clinical practice concerns the compatibility of pre-analytic stability with real life conditions of use. We, therefore, assessed the effect of drying time on the tacrolimus concentrations measured in blood tips. A minimum drying time of 24 h at room temperature has frequently been reported [15–18]. However, a shorter drying time (2-3 h at RT) did not seem to affect tacrolimus quantification in VAMS tips in the studies by Koster *et al.* and Kita *et al.* [14,16], whereas Vethe *et al.* reported lower recovery rates for VAMS tips dried for 3 h rather than 24 h at RT

[15]. Nevertheless, short drying times may be an advantage in laboratory workflows, facilitating the daily preparation of QCs and standards. We, therefore, investigated the impact of short blood drying times on the tacrolimus concentrations obtained. We found that tip drying could be accelerated by heating to 37 °C for 30 min in a controlled-temperature chamber. Like Kita *et al.*, we also found that tacrolimus could be extracted and accurately quantified from tips dried for only 2 h at RT.

Besides, conflicting results for the medium- and long-term stability of tacrolimus in microsamples have been reported. Kita *et al.* reported that tacrolimus was stable for only 43 h at RT, with a decrease in drug concentration of more than 15% by day 3, whereas Koster *et al.* and Vethe *et al.* reported tacrolimus stability in VAMS blood stored at RT for 1 month and 2 months, respectively [14–16]. We found that samples were stable at RT for seven days, but that tacrolimus concentration had decreased considerably after one month of storage at RT. For TDM with sample collection by patients at home, sample stability is crucial, because the patients must send their samples by post for testing. Samples may not, therefore, be analyzed until several days after collection. Stability over a period of seven days may be sufficient for samples shipped by regular mail. The differences in stability reported by different authors may reflect differences in recovery. Indeed, each team uses a different mixture to extract tacrolimus from tips, potentially resulting in different recovery rates from blood samples dried over a period of days. Discrepancies in long-term stability may also reflect differences in the sealing of the storage bag and the desiccant used, because these elements are not standardized between laboratories. Moreover, one of the strengths of our stability study is the confirmation that tacrolimus levels remain stable in samples stored in conditions mimicking the sample being left in a mail box for a few days under extreme weather conditions. Like Koster *et al.*, we found that samples could tolerate high temperatures (i.e. 60 °C or 50 °C) for two days, and, like Kita *et al.*, we observed no decrease in drug concentration in samples stored at 4 °C for two days [14,16]. The long-term stability of frozen samples was also confirmed after one month of storage, consistent with the findings of Koster *et al.* Thus, VAMS devices can be used for clinical studies in which the samples need to be stored in the laboratory before analysis. A limit of our stability study is the lack of data regarding medium-term stability at intermediate storage conditions

(e.g. 1 month at +4°C at -20°C). Stability of tacrolimus in VAMS samples at this condition should be further evaluated.

We also conducted a clinical validation, in which we compared venous and capillary tacrolimus blood concentrations in kidney or liver transplant recipients. The correlation between these concentrations must be systematically assessed, to check that VAMS is a reliable method for tacrolimus TDM [9,19]. In this study, venous and capillary concentrations appeared to be very well strongly correlated (r of 0.97), and Bland-Altman analysis showed that the usual TDM method (liquid samples of venous blood) and the VAMS method (capillary blood) provided similar results. This good concordance may be linked to the VAMS technology, which prevented a hematocrit effect and ensured that the samples were homogeneous [20]. Indeed, other teams using the same device have also reported consistency between tacrolimus determinations on venous and capillary blood for a large range of concentrations [15,18,21]. Zwart *et al.* used another device to collect an accurate volume of blood for dried blood spots [17]. They reported good agreement between the tacrolimus AUC obtained with microsamples of capillary blood and that obtained with tubes of venous blood, but, they found a bias between the two methods when the analysis focused exclusively on trough concentrations of the immunosuppressant. Contrary to some other teams [15,17,21], we did not report a comparison between tacrolimus AUC obtained from liquid venous blood and AUC obtained from VAMS samples since the study was not design for this purpose. Based on satisfying results of the clinical validation of our assay and above mentioned literature data, we assumed that the benefit-risk balance was good enough to use VAMS for tacrolimus routine TDM. Indeed, one of the major advantages of microsampling devices for the TDM of tacrolimus is that they make it possible to obtain repeat samples from patients, in a minimally invasive manner. The estimation of tacrolimus AUC is, therefore, compatible with clinical settings. We emphasized this aspect through clinical application of the method described here in kidney transplant recipients. Trough concentration is known to be a poor surrogate marker of tacrolimus exposure, and the last consensus conference for tacrolimus TDM recommended determining individual AUC/C₀ ratios several times during patient follow-up [4]. The patients included in the clinical application of our assay benefited from this approach and we observed a wide range of AUC_{0-24h} and AUC/C₀ ratios which confirms the suboptimal correlation

between tacrolimus C_0 and AUC. Furthermore, in our study AUCs were determined for hospitalized patients. Interestingly, Gustavsen *et al.* reported that AUC could be accurately determined from samples collected at home, by the patients themselves. They also showed that the AUCs obtained with capillary microsamples were highly consistent with those obtained for venous blood samples [21]. Hence, tacrolimus determinations in VAMS microsamples could overcome the practical obstacles to the measurement of tacrolimus exposure (e.g. through AUC). Thanks to analytical methods such as that described here, it should be possible to improve tacrolimus TDM and tacrolimus response in transplant recipients and further studies should be performed to confirmed this statement.

Otherwise, unfortunately our study was not designed to provide objective data regarding practical use of VAMS in clinical setting. In particular, we did not quantify users (medical staff and patients) feedbacks, medico-economics aspects and sample quality (over or under filling of tips). This last point might be critical to assess the efficiency of person's training and to rely on measurements. An automated quality control process should be implemented whenever possible [12,22]. Future study is still needed to address these questions to fully demonstrate the reliability of VAMS strategy in routine.

The ability of our method to determine the levels of only one immunosuppressant, tacrolimus, could be seen as a limitation. Indeed, transplant recipients are almost always treated with a combination of immunosuppressive drugs for which TDM is necessary. Tacrolimus is usually the cornerstone of immunosuppression, but it would be advantageous to be able to use the same microsample-based analytical method to determine the levels of cyclosporin, m-tor inhibitors (everolimus and sirolimus) or mycophenolic acid, as reported in other studies [16–18]. We are currently working on the extension of our method to these drugs.

5. Conclusion

We developed a rapid, sensitive and accurate HPLC-MS/MS method, and validated its analytical performance. Comparison with the reference method of quantification of tacrolimus in liquid venous whole blood fulfilled clinical requirements. This assay, based on VAMS microsampling strategy, is promising to be used in the routine TDM of tacrolimus or for pharmacokinetic studies. The

implementation of microsampling strategies should be encouraged for the assessment of tacrolimus exposure and the optimization of tacrolimus response in transplant recipients. This feasible approach may constitute a crucial step forward for tacrolimus TDM and for improvements in the quality of life of transplant recipients.

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FIGURE LEGENDS

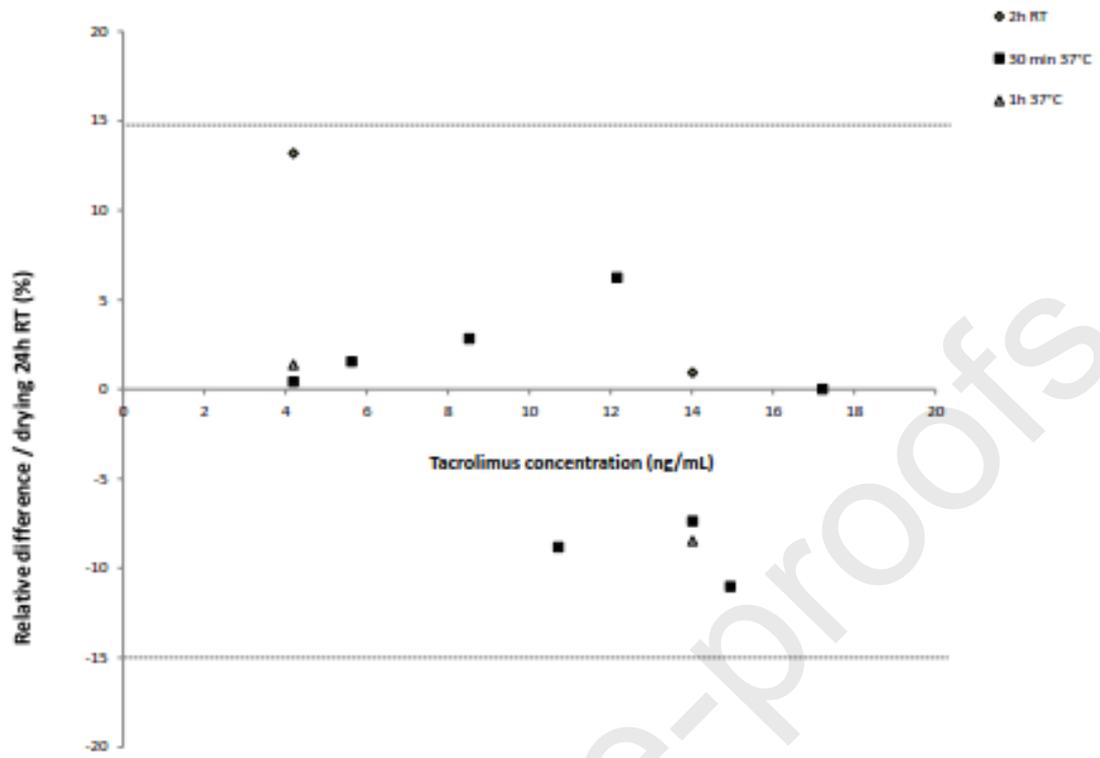
Figure 1: Influence of drying time on tacrolimus concentration in blood collected by VAMS. RT: room temperature,

Figure 2: Passing-Bablok analysis of tacrolimus (Tac) concentrations determined liquid venous blood samples and blood collected by VAMS.

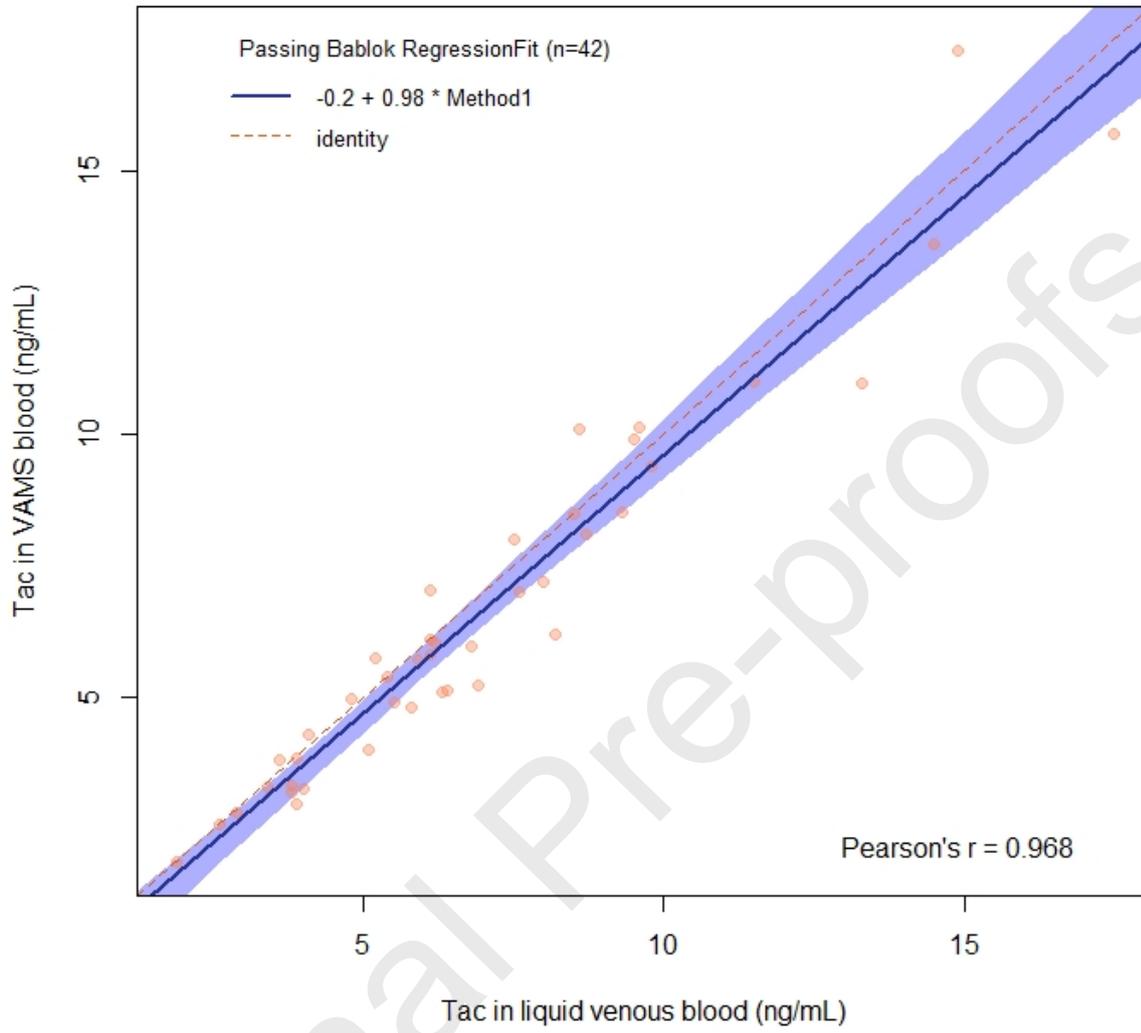
Figure 3: Bland-Altman analysis of tacrolimus (Tac) concentrations determined in liquid venous blood samples and blood collected by VAMS

Continuous lines (—) represent the mean systematic error, dotted lines (-.-) represent limit of clinical acceptance (+/- 20%), small dotted lines (- - -) represent 95% lower and upper limits of agreement

Colors: There is no need to use color to print the figures.



My Passing Bablok Regression



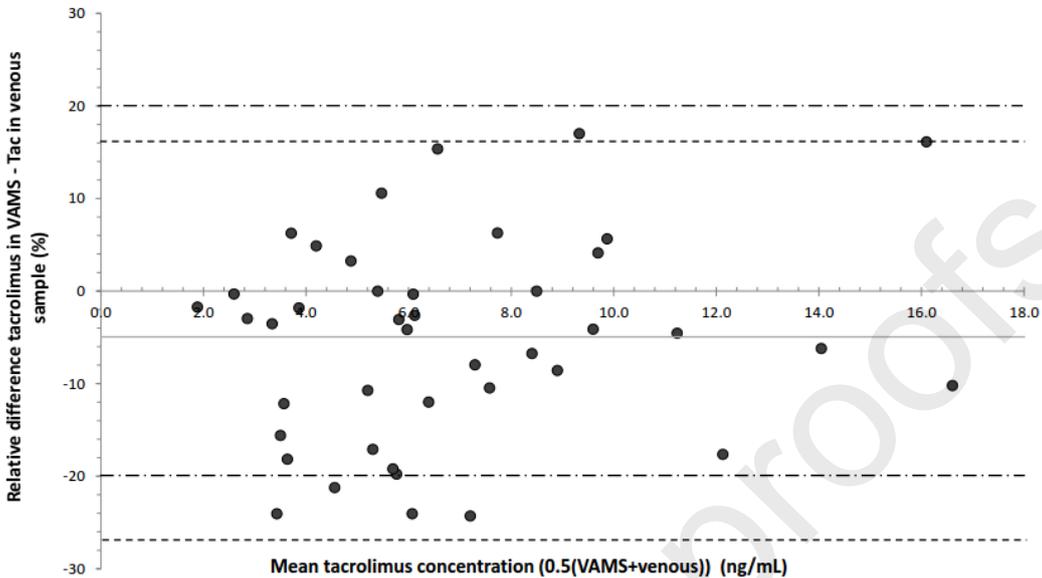


Table 1: Within day and between day accuracy and precision of tacrolimus assay in VAMS

CQ level	Within day precision (CV%) (n=5)	Between day precision (CV%) (n=15)	Within day accuracy Bias (%) (n=5)	Between day Precision Bias (%) (n=15)
LLOQ	4.0	8.0	12.9	6.0
QC1	4.7	7.4	2.7	1.2
QC2	7.7	11.6	-1.3	-6.6
QC3	5.9	6.9	-7.4	-6.5
QC4	10.2	10.2	-4.2	-4.9

QC: Quality control; CV: Coefficient of variation

Table 2: Influence of hematocrit on tacrolimus quantification in VAMS

	Concentration levels			
	2.5 ng/mL	5 ng/mL	15 ng/mL	30 ng/mL
Ht 20%	-2.5	-0.2	-9.6	-5.7
Ht 60%	3.2	1.4	-3.0	-3.7

Ht: hematocrit

Results are expressed in % as relative difference compared to concentrations measured in sample at hematocrit 40% (n=5 per condition)

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Table 3: Recovery of tacrolimus in samples of blood collected with VAMS

	Tacrolimus concentration level in whole blood (ng/mL)		
	<i>5</i>	<i>15</i>	<i>30</i>
Ht 25%	1.04	0.91	0.87
Ht 30%	0.89	0.94	0.93
Ht 40%	0.88	0.98	0.85
Ht 50%	0.91	0.87	0.81
<i>CV (%)</i>	7.6	8.9	6.9

Recoveries are expressed as are ratio of (IS normalized) LC-MS signal of tacrolimus in VAMS/ tacrolimus signal in wet sample at the same hematocrit and concentration; CV: coefficient of variation; Ht: hematocrit

Table 4: Process efficiency and matrix effect of tacrolimus assay in VAMS

Experiment 1				
Tacrolimus blood concentration	Process efficiency			Matrix factor
	Mean PE	CV PE (%)	CV-ISc (%)	CV (%)
5 ng/mL (n=6)	0.94	13.94	11.00	10.58
30 ng/mL (n=6)	0.94	4.91	9.58	5.39
Experiment 2 (tacrolimus at 6 ng/mL)				
	Mean MF	CV MF (%)	Mean ISc MF	CV ISc MF (%)
Ht 40% ^a	1.15	14.44	0.92	12.56
Ht 20% ^b	1.03		0.95	
Ht 40% ^b	1.01	3.20	1.12	9.60
Ht 50% ^b	0.97		0.97	

MF: Matrix factor; PE: process efficiency; CV: coefficient of variation; IS: internal standard; ISc: IS corrected; Ht: hematocrit

^a: n=6 sources of blood from 6 different individuals, each sample was analyzed in triplicate

^b: 1 source of blood at adjusted hematocrit, each sample was analyzed in triplicate

Samples were prepared by spiking drug-free blood from 6 different sources with tacrolimus at several concentrations. Samples were extracted according to the procedure described above (samples A). For each source, blank blood samples were extracted and post-fortified with tacrolimus and IS to reach the same concentrations than the one expected in samples A for a recovery of 100% (samples B). Neat solutions without blood matrix were prepared in duplicate by spiking tacrolimus and IS at the same concentrations than the final concentration expected in sample B (samples C). Process efficiency (PE) was calculated as follow: $PE = (\text{peak area of sample A} / \text{peak area of sample C})$. Absolute matrix effects of each source were estimated for tacrolimus and the IS by matrix factors (MF) determined as $MF = (\text{peak area of sample B} / \text{peak area of sample C})$. IS corrected MF was calculated by dividing the MF of the tacrolimus by the MF of the IS.

Table 5: Stability of tacrolimus in VAMS samples

Type of blood sample	Expected concentration (ng/ml)	Storage condition				
		7 days RT	48h 4°C	48h 60°C	1 month RT	1 month - 80°C
QC	2.61	-7.1	<i>n.t.</i>	<i>n.t.</i>	<i>n.t.</i>	<i>n.t.</i>
QC	6.96	-1.7	<i>n.t.</i>	<i>n.t.</i>	<i>n.t.</i>	<i>n.t.</i>
QC	14.6	7.2	<i>n.t.</i>	<i>n.t.</i>	<i>n.t.</i>	<i>n.t.</i>
QC	31.7	1.3	<i>n.t.</i>	<i>n.t.</i>	<i>n.t.</i>	<i>n.t.</i>
Pat	3.3	<i>n.t.</i>	4.2	-11.0	<i>n.t.</i>	<i>n.t.</i>
Pat	14.2	<i>n.t.</i>	-7.5	-3.3	<i>n.t.</i>	<i>n.t.</i>
Pat	6.00	<i>n.t.</i>	<i>n.t.</i>	<i>n.t.</i>	-37.7	5.8
Pat	2.20	<i>n.t.</i>	<i>n.t.</i>	<i>n.t.</i>	-20.9	-6.3
QC	2.73	<i>n.t.</i>	<i>n.t.</i>	<i>n.t.</i>	-21.8	-11.7
QC	15.40	<i>n.t.</i>	<i>n.t.</i>	<i>n.t.</i>	-25.0	-6.1

QC: manufactured quality control sample. The first 4 QC and the last 2 QC correspond to two different batches. The expected concentration is provided by the manufacturer according to the amount of tacrolimus weighed in the sample; Pat: patient sample; RT: room temperature; n.t.: not tested, n=3 replicate/ condition

Results are reported as bias (%) to the expected concentration

Table 6: Clinical application: monitoring of tacrolimus exposure and non-compartmental pharmacokinetic analysis in kidney transplant recipients

Tac formulation	Parameter	Tac daily dose (D) (mg)	AUC _{0-24h} (ng.h/mL)	C ₀ (ng/mL)	Ratio AUC/C ₀ (h)	Ratio AUC/D (ng.h/mL.mg)	C _{max} (ng/mL)	T _{max} (h)
Advagraf® (n=4)	median	15.3	320.2	8.1	44.9	25.5	28.8	1.5
	min	5	305.2	6.1	30.3	18.0	22.0	1.0
	max	20	526	11.5	50.4	61.3	46.1	2.0
Envarsus® (n=6)	median	8.5	350.4	8.3	39.5	44.3	24.0	6.0
	min	2.0	222.0	5.4	28.7	21.0	11.7	4.0
	max	17.0	541.9	14.9	66.0	111.0	37.7	6.0

Tac: tacrolimus; AUC: area under the curve of concentration versus time; C₀: trough concentration; C_{max}: peak concentration; T_{max}: time when C_{max} is observed; D: tacrolimus dose

**Volumetric absorptive microsampling for the quantification of tacrolimus in capillary blood by
high performance liquid chromatography-tandem mass spectrometry**

CRediT Author Statement

CT: Conceptualization, Validation, Writing-Original Draft, Visualization, Project administration. MJF: Investigation, Visualization, Writing-Original Draft. JQR: Investigation, Visualization, Writing-Original Draft. JC: Resources. PHD: Resources. MCV: Writing - Review & Editing. EB: Writing - Review & Editing. FL: Conceptualization, Writing-Original Draft, Validation, Supervision.

Volumetric absorptive microsampling for the quantification of tacrolimus in capillary blood by high performance liquid chromatography-tandem mass spectrometry

Tron et al.

Highlights:

- Volumetric absorptive micro-sampling is an innovative alternative to venipuncture
- A method was developed to measure whole-blood tacrolimus in microsamples
- The UHPLC-MS/MS method is fast, sensitive, precise and accurate
- This analytical assay was clinically validated according to a reference method
- The assay was applied to assess tacrolimus exposure in transplanted patients