Validation and evaluation of four sample preparation methods for the quantification of intracellular tacrolimus in peripheral blood mononuclear cells by UHPLC-MS/MS

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Abstract

Rejection and toxicity occur despite monitoring of tacrolimus blood levels during clinical routine. The intracellular concentration in lymphocytes could be a better reflection of the tacrolimus exposure. Four extraction methods for tacrolimus in peripheral blood mononuclear cells were validated and evaluated with UHPLC-MS/MS.

Methods based on protein precipitation (method 1), solid phase extraction (method 2), phospholipids and proteins removal (method 3) and liquid-liquid extraction (method 4) were evaluated on linearity, lower limit of quantification (LLOQ), imprecision and bias. Validation was completed for the methods within these requirements, adding matrix effect and recovery.

Linearity was 0.126 (LLOQ)-15 µg/L, 0.504 (LLOQ)-15 µg/L and 0.298 (LLOQ)-15 µg/L with method 1, 2 and 3, respectively. With method 4 non-linearity and a LLOQ higher than 0.504 µg/L were observed. Inter-day imprecision and bias were ≤4.6%, ≤10.9%; ≤6.8%, ≤-11.2%; ≤9.4%, ≤10.3% and ≤44.6%, ≤23.1%, respectively, with methods 1, 2, 3 and 4. Validation was completed for method 1 and 3 with matrix effect (7.6%; 15.0%) and recovery (8.9%; 10.8%), respectively.

The most suitable UHPLC-MS/MS method for quantification of intracellular tacrolimus was protein precipitation due to the best performance characteristics and the least time-consuming rate and complexity.

Keywords

Tacrolimus; Ultra-high-performance liquid chromatography (UHPLC) – tandem mass spectrometry (MS/MS); Peripheral Blood Mononuclear Cells; Extraction procedures; Validation; Immunosuppressors

Abbreviations

TAC: Tacrolimus; TDM: therapeutic drug monitoring; FKBP12: FK-binding protein 12; NFAT: nuclear factor of activated T cells; NF-kB: ; PBMCs: peripheral blood mononuclear cells; UHPLC-MS/MS: Ultra-high performance liquid chromatography coupled to tandem mass spectrometry; ACN: acetonitrile; DTT: dithiothreitol; MTBE: methyl *tert*-butyl ether; PTT: protein precipitation; LLOQ: lower limit of quantification; LLE: liquid-liquid extraction; EMA: European Medicines Agency; ULOQ: upper limit of quantification; MF: matrix factor.

1. Introduction

Tacrolimus (TAC) is a pivotal immunosuppressant used post solid organ transplantation [1,2]. Its exposure is characterized by a narrow therapeutic window and a high intra- and interindividual pharmacokinetic variability [3–5]. Therefore, therapeutic drug monitoring (TDM) based on the trough concentration of TAC in whole blood is mandatory in clinical practice [6]. Prevention of adverse events has a considerable role during TDM, beside achieving optimal efficacy [7].

Whole blood concentrations may not be the only tool to predict efficacy and toxicity and a different approach to monitor TAC exposure could further personalize TAC therapy [3]. This could be needed as acute rejection still occurs in 8-15% the low risk patients using TAC during the first year post transplantation despite intensive TDM [6]. Moreover, TAC exposure can lead to nephrotoxicity [8,9] and rejection and toxicity occur even while having adequate blood levels [10,11]. Additionally, in some patients low tacrolimus whole blood concentrations do not increase the risk for rejection [12].

The mechanism of action of TAC occurs inside the lymphocytes, where it inhibits the phosphatase activity of calcineurin due to the binding of intracellular FK-binding protein 12 (FKBP12) [13,14]. This results in less activation of transcription factors affecting the regulation of cytokines, such as interleukin-2, tumour necrosis factor-β and interferon-γ, and activation and proliferation of T lymphocytes. The effect of TAC is consequently a decreased immune response to the allograft [1,15].

The intracellular concentration of TAC in lymphocytes has therefore been proposed as a good tool to reflect the TAC exposure and may correlate better with clinical outcomes than whole blood concentrations [16,17]. In accordance, lower concentrations of TAC inside peripheral blood mononuclear cells (lymphocytes and monocytes, PBMCs) were associated with rejection scores on day 7 after liver transplantation [18]. Whereas the correlation between the intracellular and the whole blood concentration of TAC remains unclear [19,20].

Few articles have validated a method for the quantification of TAC in PBMCs [21–25]. The first two published methods, done by Capron A, et al [21] and Lemaitre F, et al [23,26], consisted of a liquid-liquid extraction (LLE) of TAC followed by evaporation with nitrogen gas or protein precipitation (PPT). These two methods are complicated and time-consuming extraction procedures though being sensitive. Recently, three less time-consuming validated methods have been published. Pensi D, et al [22] validated a quantification method with extraction by an online solid-phase extraction (SPE) coupled to the ultra-high performance liquid chromatography – tandem mass spectrometry (UHPLC-MS/MS) and Bahmany S, et al [24] validated a method based on magnetic separation with the use of a reagents kit containing paramagnetic beads. Unfortunately, both methods used equipment or material that may not be available in a general hospital. Lastly, a simple method based on protein precipitation (PPT) was validated by Romano P, et al [25] using generally available materials. Normally, PPT results in a sample with more noise and therefore a higher lower limit of quantification (LLOQ), however the LLOQ of the last method was comparable with the other methods.

To introduce a new method for the intracellular TAC quantification in the clinical hospital routine, it is crucial to compare the existing published validated methods. The most suitable method can be used to promote further research into intracellular TAC. This is also useful during implementation as intracellular TAC could be a better predictor for clinical outcomes [18]. The aim of this study was therefore to validate and evaluate four different extraction procedures on their compatibility for clinical practice for the quantification of TAC in PBMCs by UHPLC-MS/MS.

2. Materials and methods

2.1 Chemicals and reagents

Tacrolimus, LC-MS grade ammonium acetate, zinc sulphate heptahydrate, formic acid, Tris, dithiothreitol (DTT), ammonium hydroxide, methyl *tert*-butyl ether (MTBE), sodium chloride 0.9% and EDTA-free protease inhibitor cocktail tablets were purchased from Sigma-Aldrich (St Louis, MO, USA). Ascomycin, the internal standard (IS), was obtained from Recipe (Darmstadt, Munich, Germany). LC-MS grade acetonitrile (ACN) and LC-MS grade methanol were purchased from Merck Biosciences (Danvers, MA, USA). Ficoll-Paque plus was obtained from GE Healthcare (Uppsala, Sweden) and ammonium chloride from Stemcell Technologies (Vancouver, Canada).

2.2 Isolation PBMCs

Whole blood from renal transplant patients and buffy coats of healthy volunteers were used for isolation of PBMCs, following the same procedure previously published [27]. 4 mL of blood samples for routine clinical practice for blood tacrolimus exposure analysis were used. Briefly, isolation was carried out by Ficoll density gradient by centrifugation of SepMate^{TM} Tubes (Stemcell Technologies). After lysis of the erythrocytes, three washing steps were carried out. Cell counting was performed by Scepter 2.0 Handheld Automated Cell Counter (Merck). The pellet was resuspended in 100 µL hypotonic buffer per 2 x 10^6 cells and the solution was stored at -80 °C until analysis.

2.3 Preparation of calibrators, quality controls and internal standard

Working solutions for calibrators and quality controls (QCs) were prepared separately from different TAC stock solutions of 1 g/L in ACN. 20 µL stock solution was diluted with ACN until 300 µg/L (working solution) was achieved. From the working solution, calibrators in ACN were prepared by dilution of the previous calibrator in the range 0.126 – 15 µg/L. Four QCs were made by dilution of 15 µg/L in ACN. QCs had the following concentrations: QC1 12 µg/L; QC2 8 µg/L; QC3 1 µg/L and QC4 0.35 µg/L. Ascomycin was chosen as internal standard (IS) and was prepared using the same procedure as during

clinical routine [28]. The final working solution concentration of ascomycin in ACN was 500 µg/L. Calibrators and QCs were stored at -80 °C upon analysis. The IS working solution was stored at 4 °C until intracellular TAC determination by UHPLC-MS/MS.

2.4 Tacrolimus extraction

To determine the best and most suitable method for implementation in our hospital four different methods for TAC extraction were evaluated. Sample preparation of the calibrators and QCs started by spiking 50 µL blank PBMCs with 50 µL calibrator or QC. Preparation of patient samples started with adding 50 µL ACN, the solvent of the calibrators and QCs, to 50 µL patient PBMC sample (1 x 10^6 cells) to achieve the same conditions.

2.4.1 Method 1: protein precipitation (PPT)

PPT was an optimized method based on the validated method of Romano P, et al [25]. To 50 µL sample 50 µL of 0.1 mM zinc sulphate in water/methanol (80:20 *v/v*) and 150 µL IS were added. After vortex and centrifugation at room temperature for 8 minutes at 13.000 x *g* the supernatant was transferred into a vial for UHPLC-MS/MS analysis.

2.4.2 Method 2: Solid phase extraction (Oasis)

To perform a SPE with commonly available materials, the conditions of the validated online SPE method of Pensi D, et al [22] were mimicked using Oasis HLB 96-well µElution plate (Waters, Milford, USA). The plate was conditioned with 400 µL ACN, equilibrated with 400 µL water/ACN (90:10 *v/v*) and 50 µL sample, premixed with 25 µL IS and 150 µL ultrapure water, was added. Two washing steps were performed with first 100 µL water/ACN (90:10 *v/v*) and then 100 µL water. Samples were eluted using 200 µL ACN and transferred into a vial for analysis.

2.4.3 Method 3: Pass-through sample preparation (Ostro)

The third extraction was a non-validated method using Ostro™ Pass-through Sample Preparation (Waters) for removal of proteins and phospholipids. IS (150 µL) was added to 50 µL sample and after vortex, the solution was transferred to the Ostro plate. The sample was filtered by applying 15 mmHg of vacuum for 5 minutes. The filtrated solution in the recollection plate was measured by UHPLC-MS/MS.

2.4.4 Method 4: Liquid-liquid extraction (LLE)

For the LLE, the method of Capron A, et al [21] was modified. Solvents (25 µL IS, 500 µL sodium chloride 0,9% solution, 200 µL 2M ammonium hydroxide and 1 mL MTBE) were added to 50 µL sample in a glass tube. The solution was vortexed strongly, centrifuged at room temperature for 5 minutes at 1500 x *g* and the upper layer was evaporated with nitrogen. The residue was reconstituted in 200 µL ACN and transferred into a vial for analysis.

2.5 UHPLC-MS/MS conditions

By ensuring the possibility for implementation in clinical practice the same UHPLC-MS/MS conditions were used as during the clinical routine quantification of immunosuppressors in whole blood [28].

2.6 Validation

To compare the four different extraction protocols, the methods were tested for three days considering linearity, inter- and intraday imprecision and bias and sensitivity. Afterwards, the methods fulfilling most of the requirements were extensively validated for five days, adding matrix effect and recovery analysis to better define the differences between those methods to be implemented at our hospital. Carry-over, dilution integrity and stability studies were also performed. Validation was performed following European Medicines Agency (EMA) guidelines [29].

2.6.1 Calibration curve

The calibration curve consisted of calibrators in the range 0.126 – 15 µg/L, a blank sample (without tacrolimus and IS) and a zero sample (with IS and without tacrolimus). The final point of the curve was considered as the upper limit of quantification (ULOQ). The calibration curve was prepared each day with every method and was analysed at least three times for three days. The calibration curves were generated by linear fit of the TAC/IS area response ratio multiplied by IS concentration versus TAC concentration (no weighting; excluding the option to force through the point of origin), using the TargetLynx™ v4.1 software (Waters). The curve was considered linear at $R^2 > 0.995$ and when 75% of the calculated calibrator concentrations were within ±15% of the nominal value, except for the LLOQ for which it was within ±20%.

2.6.2 Lower limit of quantification

The lowest points of the curve (between 0.126 and 0.504 µg/L) were analysed to evaluate the LLOQ per method, defined as the lowest point within the requirements of linearity (within ±20%), inter- and intraday bias (relative bias (δ_r) within ±20%) and imprecision (coefficient of variation (*CV)* ≤20%) and signal to noise (S/N) (>5).

2.6.3 Imprecision and bias

Inter- and intra-day imprecision and bias were evaluated using QCs. The intraday imprecision and bias were evaluated by measuring five times the QC in a single run. For inter-day analysis, QCs were measured at least three different days for all the methods. According to the EMA guidelines [29], the bias (δ_r) should be within ±20% and *CV* for imprecision should not exceed 15%.

2.6.4 Matrix effect and recovery

The matrix effect and recovery were evaluated using TAC-free PBMCs from different donors. The blank matrix of each individual donor was spiked pre-extraction or post-extraction with QC1, QC2, QC3 or IS. Furthermore, QC1, QC2, QC3 and IS were analysed without blank matrix. The matrix effect was evaluated by calculating for each individual donor the matrix factor (MF) consisting of the ratio of the peak of post-extraction samples to the peak without matrix. The *CV* of the IS-normalised MF should be

≤15%. The recovery was evaluated by analysis of QC1, QC2 and QC3 spiked pre-extraction and post-extraction and the *CV* of IS-normalised recovery of the mean of all QCs should be ≤15%.

2.6.5 Carry-over

Carry-over was assessed by injecting three blank samples after a high concentration sample at the ULOQ (15 µg/L). Carry-over was accepted if the peak area response in the blank samples obtained after measurement of the high concentration sample was ≤20% of the analyte (TAC) peak area response at the LLOQ and ≤5% of the peak area response of the IS.

2.6.6 Dilution integrity

To evaluate the dilution integrity, five replicates of a blank PBMC sample were prepared at two times the ULOQ (30 µg/L) and subsequently diluted 1/10 with blank PBMCs to a concentration of 3 µg/L. Imprecision should be ≤15% and bias should be within ±15%.

2.6.7 Stability

Stability studies included short-term stability of TAC extracted samples, using replicates of QC1 and QC3, and long-term stability of TAC in PBMCs. Short-term stability of TAC in PBMCs after extraction at -80 °C, 4 °C and 15 °C (autosampler temperature) was evaluated after 24 and 48 or 72 hours and 1 week. Stability of TAC in PBMCs was evaluated by measuring the TAC concentration in three patient samples within 24 hours after isolation and after 1, 2, 3 and 4 weeks. The samples were considered as stable when the mean concentration was within ±15% of the nominal concentration, according to the EMA guidelines [29].

2.7 Biological samples

Five biological samples of patients taking TAC were extracted by the four methods after isolation of PBMCs and quantified using the calibration curve of the corresponding method to evaluate the difference in quantification with the methods. Intracellular TAC concentrations were expressed as pg/million cells. The following equation was used to calculate the intracellular concentration using the calibration curve: [X µg/L = X pg/µL → X pg/µL * 50 µL/million cells = X pg/million cells]. Whole blood

concentration of TAC was also measured following the clinical routine [28]. High, intermediate and low whole blood concentration of tacrolimus were selected of patients following routine care tacrolimus quantification, considering the target concentration in our hospital of 5-10 µg/L. Furthermore, to study different TAC concentrations after drug intake, a pharmacokinetic profile of whole blood and intracellular TAC were performed at different times: pre-dose, 1, 2, 4, 8, 12 in one transplant recipient sample receiving TAC twice-daily formulation.

3. Results

3.1 Validation

Under the chromatographic conditions described by Rigo-Bonnin R, et al [28] for UHPLC-MS/MS method, the retention time for TAC and ascomycin was 1.00 min. The UHPLC-MS/MS run time was 2.5 min, including the time necessary for the solvent gradient to return to baseline conditions before the next injection. Typical chromatograms are shown in Figure 1.

3.1.1 Calibration curve

All curves with four methods (Figure 2) had a linear fit of R^2>0.995 on each day, except for one curve with LLE (R^2=0.993). The curves of PPT and Ostro were linear in the range 0.126 – 15 µg/L, fulfilled all requirements for linearity (>75% of calibrators within ±15% of nominal value) and had a mean correlation coefficient (R^2) of >0.999 during validation. With Oasis the curve fulfilled linearity requirements in the range 0.504 – 15 µg/L and with LLE in this range calibrators and LLOQ were not within requirements of linearity.

3.1.2 Limit of quantification

Chromatograms of the lowest concentration (0.126 µg/L) with four methods are shown in Figure 1. With PPT, Oasis and Ostro the LLOQ was respectively 0.126, 0.504 and 0.298 µg/L fulfilling requirements for imprecision and bias (Table 1), linearity (within ±20%) and S/N (>5). With LLE 0.504 µg/L was not within requirements of imprecision, bias (Table 1) and linearity (>20%), despite being within requirements of S/N (>10).

3.1.3 Imprecision and bias QCs

Results of imprecision and bias of QCs are shown in Table 1. QCs were within requirements with PPT, Oasis and Ostro and were not within requirements with LLE.

3.1.4 Matrix effect and recovery

The matrix effect was within the requirements with PPT and Ostro (*CV* of IS-normalised MF 7.6% and 15.0%, respectively) and it was well compensated by the IS. Recovery was within the requirements with both methods (*CV* of IS-normalised recovery 8.9 and 10.8% with PPT and Ostro, respectively).

3.1.5 Carry over

The methods were considered as carry over-safe as carry over did not exceed 20% of the area of 0.126 µg/L TAC or 5% of the area of IS (12.5 and 13.4% of the LLOQ and 0.1 and 0.2% of the IS for PPT and Ostro, respectively).

3.1.6 Dilution integrity

After dilution the obtained results were within the requirements of ≤15% (*CV* 5.4%).

3.1.7 Stability

After extraction with both PPT and Ostro TAC was stable for at least 1 week at 15 °C, 4 °C and -80 °C (Table 2). TAC was stable for at least 4 weeks in lysate of PBMCs at -80 °C (<7.2%).

3.2 Biological samples

Table 3 showed the intracellular TAC quantification of biological samples (n= 5) with PPT and Ostro extraction methods. These samples were related with corresponding whole blood concentration. Moreover, the extensive TAC sampling showed an intracellular pharmacokinetic profile with a TAC pre-dose concentration of 9.65 ng/ml and a maximum peak of TAC concentration (C_{max}) of 25.85ng/ml at 2 hours. These results were in parallel with those obtained in whole blood pharmacokinetic profile. Different whole blood and intracellular TAC concentrations during the first twelve hours were shown in Figure 3.

4. Discussion

This article presents for the first time to our knowledge an evaluation of various sample preparation methods to quantify TAC concentration in PBMCs. As TAC inhibits calcineurin inside the PBMCs, quantification of intracellular concentrations could give a better impression of the exposure to TAC than whole blood concentrations. Therefore, monitoring the intracellular TAC concentration could potentially improve clinical outcomes post-transplantation. Although research suggests that the TAC PBMC concentration is predictive for acute graft rejection short-term [18], more research on this subject is needed to further establish the benefits of monitoring TAC in PBMC for optimizing TAC.

We started validation of four methods for intracellular TAC measurement. Three methods were based on published validated methods. PPT method differed from the published method of Romano P, et al [25] on the volumes of the used solvents. We added less solution of zinc sulphate and ACN leading to a higher signal of the analyte measured with MS. SPE with Oasis differed from the published methods of Pensi D, et al [22] and Bahmany S, et al [24] due to no availability of used materials (online SPE and Reagents Kit). The SPE conditions of Oasis differed from the first method on proportion of water and ACN during the equilibration and washing steps and elution was performed with ACN instead of mobile phase. Lastly, Capron A, et al [21] carried out LLE with different solvents during extraction (1-chlorobutane vs MTBE) and reconstitution (mobile phase vs ACN). Regarding the internal standard, ascomycin was the first choice for intracellular TAC analysis as ascomycin is used during clinical routine and most authors also used it [21,25,26].

During the validation procedure different analytical characteristics and studies were evaluated. Conditions of UHPLC-MS/MS were the same used for blood TAC measurement to simplify methodological conditions to be applied in the clinical routine. The existing published methods used comparable UHPLC-MS/MS conditions as used in our study. However some authors describe optimized conditions for TAC quantification to improve TAC measurement, thus not using the same conditions as for blood TAC [24]. More specific could be the quantification of TAC in subsets of lymphocytes (T CD4

+ and B CD19 + lymphocytes) as demonstrated by Romano P, et al [25]. However, this is more time-consuming and not applicable for implementation during clinical routine in a general hospital.

Comparing the four methods, LLE was the most imprecise and biased method, despite having a low background noise. Probably, the complex and multi-steps LLE procedure is more sensitive to influences like conditions of vortex applied before extraction, manual separation of liquid phases after extraction or nitrogen flow during evaporation. PPT, Oasis and Ostro were more sensitive and PPT was the most accurate and precise method, followed by Ostro. Although we expected more sensitivity with LLE and Oasis, the limit of quantification was lower in PPT (0.126 µg/L) and LLE or Oasis did not achieve inferior concentrations. In contrast with the results of Capron A, et al [21], who validated a LLE assay with a sensitivity of 0.010 µg/L with a sufficient accuracy and precision, our LLE extraction method reached higher imprecision in concentrations below 0.504 µg/L. If we would have improved UHPLC-MS/MS conditions, we could possibly obtain better results with LLE. However, we prioritised to use the same UHPLC-MS/MS conditions as measuring whole blood TAC levels, so our method could easily be implemented in the clinical routine of immunosuppressors TDM.

Oasis was comparable with the validated assay of Pensi D, et al [22] who validated an automated online SPE with a sensitivity of 0.0195 ng/6 million cells and linearity between 0.039 – 5 ng/6 million cells. As we mimicked the same conditions, we could not obtain equal results on sensitivity, imprecision and bias, presumably due to the lack of an automated system or introduction of human error. Furthermore, Oasis quantified a higher concentration of biological sample, compared to PPT and Ostro, which quantified similar concentrations for the biological samples. In this sense, Oasis over-quantified TAC intracellular concentrations compared with other methods, but this effect was more accentuated when intracellular concentrations of TAC were higher.

Compared with current literature, the intracellular concentrations measured with our methods were equal to previous published concentrations. Capron A, et al [21] measured 162 biological samples within the range 6 - 179 pg/million cells corresponding with whole blood concentrations within 4.9 – 14.8 µg/L. Furthermore, Lemaitre F, et al [23] reported a intracellular TAC range of 5 – 150 pg/million

cells corresponding with WB 4.3 – 18.3 µg/L. These results were in line with our intracellular TAC range of 6.5 – 64.1 pg/million cells with PPT, corresponding with whole blood TAC concentrations of 3.9 – 13 µg/L. Although, one of the samples showed whole blood TAC concentration of 2.3 µg/L and its intracellular TAC concentration was lower than our LLOQ. This result suggest that despite our high sensitivity reached with the PPT extraction method, samples of patients with low whole blood TAC concentrations could not be quantified with our method.

The relationship with blood concentrations and dose remains unclear with reports demonstrating a significant as well as a non-significant relation [19,20,23,25]. Comparing five intracellular TAC concentrations with whole blood TAC concentrations, the relation seems to be non-linear. However, the use of a higher number of biological samples is needed to establish with certainty the type of correlation. Furthermore, in our hospital the therapeutic range for whole blood TAC is defined lower (5-10 µg/L) compared to most of other European countries (10-15 µg/L). At higher concentrations the differences between methods were higher. This should be considered targeting a higher range. Moreover, the intracellular pharmacokinetic profile related to whole blood profile was similar to previous data obtained from Bahmany S*, et al* [24] who also observed an intracellular TAC C_{max} around 2 hours after drug intake.

After analysing results of the four methods on linearity, sensitivity, imprecision and bias, Oasis and LLE were not within several requirements based on the guidelines of EMA [29]. On the other hand, LLE and Oasis required more time for processing the samples, thus being less applicable for implementation in clinical routine. Oasis and LLE were therefore excluded from validation and PPT and Ostro continued with validation.

During exhaustive validation of PPT and Ostro carry-over, recovery, matrix effect and stability showed similar results within the requirements of the EMA guidelines [29]. Even as we expected Ostro to be more sensitive, LLOQ was 0.126 µg/L in PPT compared with 0.298 µg/L in Ostro. Since intracellular TAC concentrations are lower than whole blood concentration, the sensitivity of the method was an essential criterion. Although PPT is probably the extraction method most vulnerable to matrix effects,

surprisingly, it presented matrix effect values within the requirement (7.6%), meanwhile Ostro values were just within requirements (15.0%). The use of ascomycin as IS compensated the existing ion suppression observed in the TAC measurement. Romano P, et al [25] were able to measure the intracellular TAC concentration in the range of 0.115 – 5.2 µg/L with their validated PPT assay. In accordance, we validated a PPT with a comparable sensitivity (0.126 µg/L). As we optimized the PPT method, we were able to use less volume of blood (3 mL vs 40 mL), less volume of used solutions and less amount of PBMCs (1 million cells versus 6 million cells). The Ostro assay could not be compared with literature as this was the first time that Ostro was used for determination of intracellular TAC.

5. Conclusion

Using the precipitation method we developed, the implementation of quantification of TAC in PBMCs is accessible in a general hospital. We would recommend protein precipitation extraction for quantification of tacrolimus concentration in PBMCs as this was the most sensitive, quick and simple method compared to solid phase extraction (Oasis), pass-through sample preparation (Ostro) and liquid-liquid extraction. The measurement of intracellular TAC exposure could lead to a more personalized approach during TAC therapy and improve clinical outcomes.

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FIGURES AND TABLES LEGENDS CAPTIONS

Figure 1: chromatograms of TAC (A,D,G,J) at the LLOQ, IS (B,E,H,K) and blank sample (C,F,I,L) with four methods (ABC: PPT; DEF: Oasis; GHI: Ostro; JKL: LLE).

Figure 2: Calibration curves with (A) PPT; (B) Oasis; (C) Ostro and (D) LLE with 7 calibrators and 4 QCs on a random chosen day.

Figure 3. Intracellular and whole blood TAC pharmacokinetic profiles of one transplant recipient receiving twice-daily formulation of TAC at 6 different timepoints (0, 1, 2, 4, 8 and 12 hours).

Table 1: intra- and inter-day precision of QCs and LLOQ quantified after extraction with four different methods (PPT, Oasis, Ostro and LLE) against corresponding curve. *: not within requirements; NA: not applicable.

Table 2: Results of stability of QC1 (12.0 µg/L) and QC3 (1.00 µg/L) after extraction with PPT or Ostro compared to concentration at day 0. Replicates at autosampler temperature (15 ºC) were measured after 1, 2 and 7 days and replicates at 4 ºC and -80 ºC after 1, 3 and 7 days.

Table 3: Quantification of TAC concentrations in PBMCs of five tacrolimus samples with PPT and Ostro extraction methods compared with their corresponding whole blood TAC concentrations. NM: not measurable due to concentration below LLOQ.

FIGURE 1

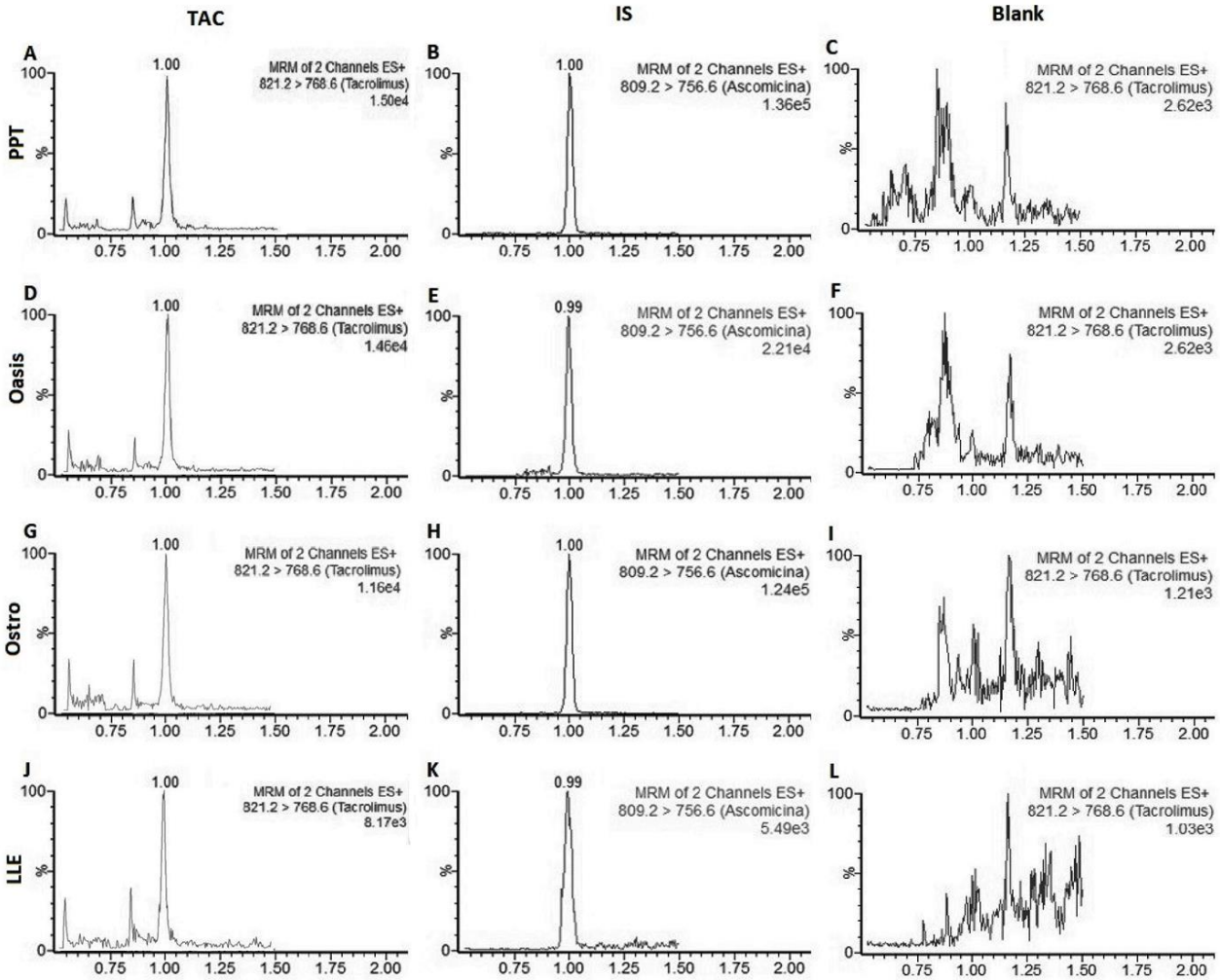
TAC
IS
Blank
A
B
C
PPT
D
E
F
Oasis
G
H
I
Ostro
J
K
L
LLE
MRM of 2 Channels ES+
821.2 > 768.6 (Tacrolimus)
1.50e4
809.2 > 756.6 (Ascomicina)
1.36e5
2.62e3
1.46e4
2.21e4
2.62e3
1.16e4
1.24e5
1.21e3
8.17e3
5.49e3
1.03e3
1.00
0.99
100
%
0
0.75
1.00
1.25
1.50
1.75
2.00

FIGURE 2

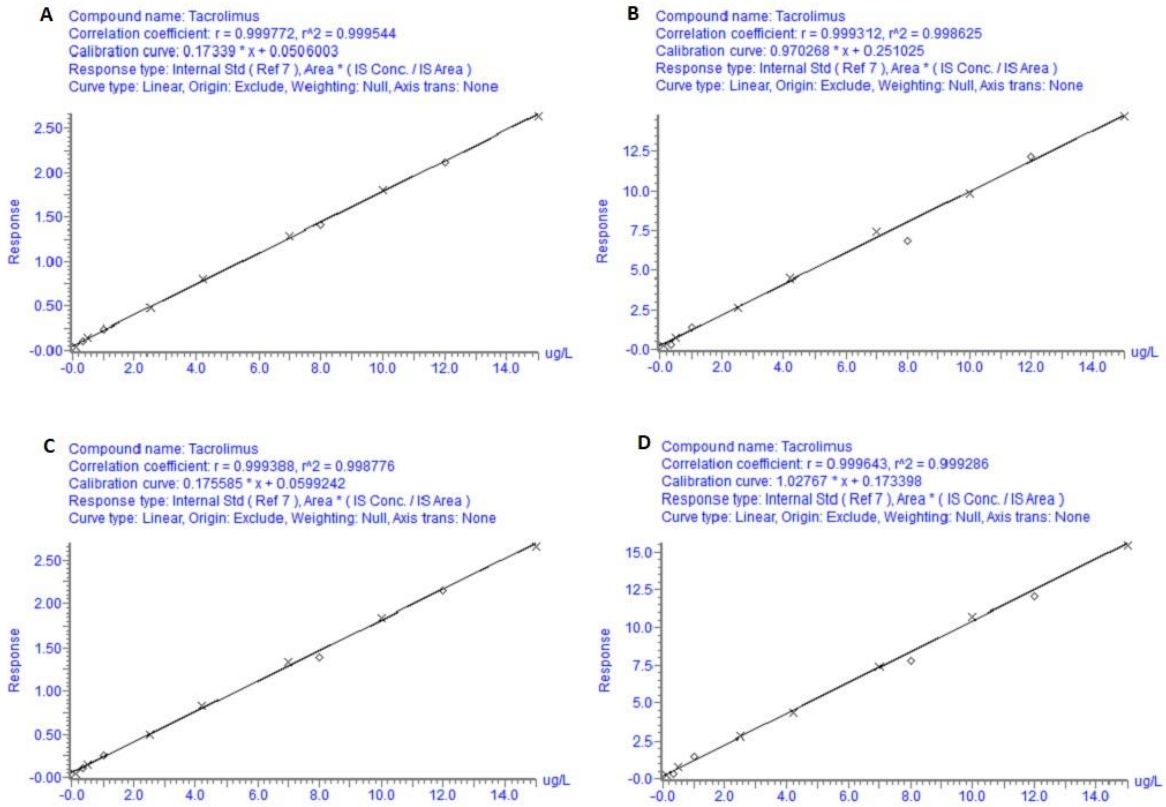
A
Compound name: Tacrolimus
Correlation coefficient: r = 0.999772, r^2 = 0.999544
Calibration curve: 0.17339 * x + 0.0506003
Response type: Internal Std (Ref 7), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None
Response
ug/L
B
Compound name: Tacrolimus
Correlation coefficient: r = 0.999312, r^2 = 0.998625
Calibration curve: 0.970268 * x + 0.251025
Response type: Internal Std (Ref 7), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None
Response
ug/L
C
Compound name: Tacrolimus
Correlation coefficient: r = 0.999388, r^2 = 0.998776
Calibration curve: 0.175585 * x + 0.0599242
Response type: Internal Std (Ref 7), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None
Response
ug/L
D
Compound name: Tacrolimus
Correlation coefficient: r = 0.999643, r^2 = 0.999286
Calibration curve: 1.02767 * x + 0.173398
Response type: Internal Std (Ref 7), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: Null, Axis trans: None
Response
ug/L

FIGURE 3

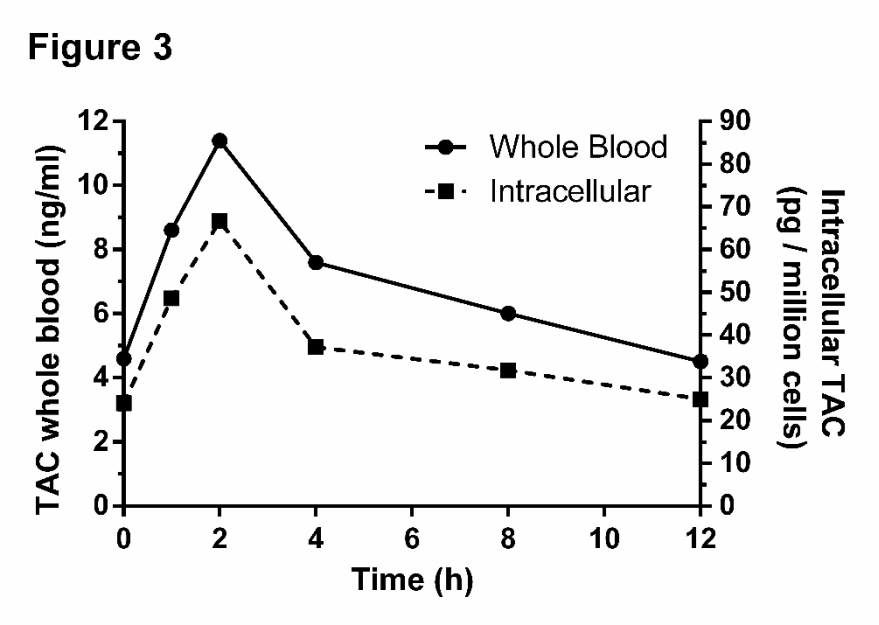
Figure 3
TAC whole blood (ng/ml)
Intracellular TAC (pg / million cells)
Whole Blood
Intracellular
Time (h)
0
2
4
6
8
10
12
0
2
4
6
8
10
12
0
10
20
30
40
50
60
70
80
90

TABLE 1

		Concentration (µg/L)	Intra-day		Inter-day	
			Imprecision (*CV*, %)	Bias (δ_r, %)	Imprecision (*CV*, %)	Bias (δ_r, %)
1: PPT	QC1	12.0	1.8	5.4	1.3	6.4
	QC2	8.00	1.6	5.9	0.1	5.8
	QC3	1.00	2.8	2.8	3.7	1.4
	QC4	0.350	4.6	10.9	4.5	10.5
	LLOQ	0.126	15.8	-8.8	18.2	-17.0
2: Oasis	QC1	12.0	2.0	2.5	2.6	-0.4
	QC2	8.00	3,6	-11,2	5.1	-3.6
	QC3	1.00	3,3	6,0	6.8	9.5
	QC4	0.350	NA	NA	NA	NA
	LLOQ	0.504	4.0	-14.8	12.0	2.1
3: Ostro	QC1	12.0	1.7	5.7	1.2	4.2
	QC2	8.00	3.1	7.2	2.0	6.5
	QC3	1.00	4.2	0.4	6.3	2.8
	QC4	0.350	7.1	10.3	9.4	9.7
	LLOQ	0.298	14.8	5.5	14.7	-0.5
4: LLE	QC1	12.0	5.0	-0.1	15.4*	-0.2
	QC2	8.00	5.1	-12.7	44.1*	-10.5
	QC3	1.00	5.4	23.1*	44.6*	-14.7
	QC4	0.350	NA	NA	NA	NA
	LLOQ	0.504	16.6	9.3	83.1*	-31.9*

TABLE 2

Method	Time	15 ºC		4 ºC		-80 ºC	
		QC1	QC3	QC1	QC3	QC1	QC3
PPT	1 days	-2.6%	-3.0%	-0.8%	-7.5%	0.7%	-6.2%
	2/3 days	1.3%	-1.4%	1.9%	-2.9%	1.5%	0.5%
	7 days	-1.8%	-5.7%	-3.7%	-6.1%	-3.3%	-9.2%
Ostro	1 days	-0.4%	-1.8%	-0.2%	-3.9%	-4.0%	-4.1%
	2/3 days	1.4%	1.0%	0.4%	-0.2%	-0.5%	-0.9%
	7 days	-5.9%	-12.6%	-3.6%	-0.8%	-0.2%	0.1%

TABLE 3

Sample	TAC whole blood concentration (µg/L)	Intracellular TAC concentration (pg/million cells)	
		PPT	Ostro
1	2.3	NM	NM
2	3.9	13.3	17.0
3	6.2	6.5	11.5
4	11.6	12.3	14.2
5	13	64.1	68.9