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Measurement of calcineurin activity in peripheral blood mononuclear cells by ultra-high performance liquid chromatography-tandem mass spectrometry. Renal transplant recipients application (pharmacodynamic monitoring)

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ABSTRACT

Background: Therapeutic drug monitoring of calcineurin inhibitor, tacrolimus (TAC), is routinely used in post-transplantation. Currently, measurement of calcineurin activity has been proposed as a promising clinical tool to evaluate efficacy and to optimize drug dosing. The main aim of our study was to develop a method to measure phosphatase calcineurin activity (CNA) in peripheral blood mononuclear cells (PBMCs) by ultra-high performance liquid chromatography-tandem mass spectrometry and to validate it following FDA and EMA guidelines.
Methods: This methodology is based on monitoring the Ca^{2+}-dependent dephosphorylation of a phosphopeptide substrate. CNA was evaluated in 5 healthy volunteers and in 5 renal transplant patients receiving twice-daily formulation of TAC before drug intake. Moreover, we studied pharmacodynamic effect of TAC and blood concentrations of TAC in different drug dose intervals (0, 1, 3, 6 and 12 h).
Results: Our results showed linearity in the range 0.04–2.00 μM with a lower limit of quantification of 0.04 μM. Coefficients of variation and absolute relative biases were < 4.3% and 10.3%, respectively. The mean recovery for peptide was 91.6 ± 4.0%. Matrix effect study displayed ion suppression, and no carry-over and interferences were observed. There were no differences in CNA between healthy and TAC-treated patients. Furthermore, CNA showed maximum inhibition at 1 h after drug intake when TAC reached the highest blood concentration.
Conclusions: This method improves the extraction phase of PBMCs and achieves faster determination compared to other techniques, bringing us closer to be applied in daily laboratory practice.

1. Introduction

Tacrolimus (TAC) is the cornerstone calcineurin inhibitor (CNI) most commonly used for the prevention of graft rejection after kidney transplantation. TAC exerts its immunosuppressive effects by inhibiting the phosphatase activity of calcineurin (CN) after binding to the intracellular FK-binding protein 12 (FKBP12) [1]. This enzyme is crucial in regulating nuclear factor of activated T-Cells (NFAT), a key transcription factor in immune response [2,3]. TAC is characterized to show a narrow therapeutic window and a high degree of intra and inter-individual pharmacokinetic (PK) variability. This wide inter-patient variability renders PK drug monitoring a requirement by usually

Abbreviations: TAC, tacrolimus; CNI, calcineurin inhibitor; CN, calcineurin; FKBP12, FK-binding protein 12; NFAT, nuclear factor of activated T-cells; PK, pharmacokinetic; PD, pharmacodynamic; CNA, calcineurin activity; PBMCs, peripheral blood mononuclear cells; CsA, cyclosporine; RII, subunit of protein kinase A; RIIp, phosphopetide substrate derived from subunit of protein kinase A; UHPLC-MS/MS, ultra-high performance liquid chromatography mass spectrometry; RII-IS, leucine stable isotope-labeled form of subunit of protein kinase A; DTT, dithiothreitol; BSA, bovine serum albumin; OA, okadaic acid; SC, sanguinarine chloride; QC, quality control; ACN, acetonitrile; TFA, trifluoroacetic acid; MS/MS, mass spectrometer; MRM, multiple reaction monitoring; FDA, Food and Drugs Administration; EMA, European Medicine Agency; CV, coefficient variation; δ_r, relative bias; LLOQ, lower limit of quantification; S/N, signal-to-noise

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measuring TAC blood trough levels [4,5]. Other strategies for drug monitoring, including pharmacodynamic (PD) monitoring may be useful for an improved assessment of drug effect on enzymatic targets beyond the measurement of TAC concentrations. TAC PD monitoring in CN activity (CNA) assays, directly quantifies the effect of CNIs on their target enzyme and could be an excellent measure to reflect its biological effect [6,7].

Different clinical studies have demonstrated that CNI diminished CNA after drug intake in solid organ transplantation measured in whole blood, leukocytes and peripheral blood mononuclear cells (PBMCs) [8–10]. Transplant patients receiving cyclosporine (CsA) showed less CNA with maximum inhibition after 2 h of drug intake [8,11–15]. In contrast, patients with TAC treatment diminished CNA after 1 h after oral administration [9,12,14,16,17]. Few studies performed in small groups of kidney and liver transplantation patients have observed that despite showing optimal TAC exposure, CN was less inhibited in patients with acute rejection [6,18–21]. Also, a two year prospective study revealed that patients with a CNA over 102 or below 12 pmol·min^{-1}·mg^{-1} were associated to rejection events [22]. These results evidence the lack of relationship between CNA and TAC blood concentrations. Lack of strong correlations between TAC levels, CNA and clinical events related to therapeutic efficacy or toxicity in renal transplantation still remains unclear [23].

Previous bioassays reported the impact of TAC on CNA and cytokine synthesis [20,24]. Several methods using radiometric, spectrophotometric or fluorimetric detections have been described to indirectly measure CNA [10,11,25–28]. These methods are based on monitoring the Ca^{2+}-dependent dephosphorylation of a phosphopeptide substrate derived from the RII subunit of protein kinase A (RIIp). Ultra-high performance liquid chromatography mass spectrometry (UHPLC-MS/MS) is the most sensitive and selective detection method, but only the study by Carr L, et al. reported a method based on this technology [29]. Some shortcomings related to the validation procedure (e.g., selectivity, carry-over, recovery and matrix effect) had not been evaluated in previous study. Indeed, these analytical techniques to measure CNA are complex, time consuming and expensive.

In this study, our aims were to develop a new, rapid, specific and feasible UHPLC-MS/MS method for measuring CNA based on the monitoring of RIIp peptide dephosphorylation in PBMC and to validate it in order to be applied to renal transplant patients.

2. Materials and methods

2.1. Chemicals and reagents

19-aminoacid peptide, RII (DLDVPIPGRFDRRVSVAAE; 2112.39 g·mol^{-1}), and a serine phosphorylated form of the RII peptide, RIIp (DLDVPIPGRFDRRVpSVAAE; 2192.39 g·mol^{-1}) were synthesized by GenScript. Leucine stable isotope-labeled form, RII-IS (D[$^{13}C_6{}^{15}N$] LDVPIPGRFDRRVSVAAE, 2119.39 g·mol^{-1}) was obtained from Eurogentec. RII was used to prepare calibration and control materials, RIIp as a CN substrate and RII-IS as the RII internal standard.

2.2. Buffers preparation

A lysis buffer containing 50 mM Tris (pH 7.5), 0.50 mM dithiothreitol (DTT) and EDTA-free protease inhibitor cocktail (1 tablet per 10 mL of buffer) was used for PBMCs lysis after their isolation. A reaction buffer composed by 20 mM Tris (pH = 8.0), 0.50 mM DTT, 100 mM NaCl, 0.10 g·L^{-1} BSA, 125 nM okadaic acid (OA), 12 μM sanguinarine chloride (SC), 1 mM $CaCl_2$ and EDTA-free protease inhibitor cocktail was prepared for phosphatase activity. Finally, an inhibitor solution containing the same as reaction buffer using 50 mM EGTA instead of $CaCl_2$ was arranged. All these buffers were replaced every three months and DTT, OA, SC, BSA and protease-inhibitors were added on the day of analysis.

2.3. Blood samples collection and PMBCs isolation procedure

Whole blood samples were collected into 3 mL-tubes with EDTA-K_3 from patients. After the extraction, the tubes were stored at 4 °C. Blank PBMCs were isolated from buffy coats from healthy volunteers. All patients provided written informed consent.

PBMCs were isolated using density gradient by Ficoll into SepMate™ tubes (Stemcell Technologies). Tubes were centrifuged for 10 min at 1200*g* at 10 °C. The top layer, which contains PBMCs, was washed with cold PBS and centrifuged for 8 min at 600*g* at 10 °C. The pellet was resuspended in 500 μL of cold PBS and 3 mL of NH_4Cl solution (130 mM) and incubated into ice for 5 min to lyse erythrocytes. After centrifugation for 5 min at 600*g* at 10 °C, harvested cells were washed with cold PBS. It is very important to ensure that red cells were lysed, so if not it is necessary to repeat lysis process indeed increasing the volume of NH_4Cl solution. Cells were suspended in 200 μl of PBS. Afterwards, 5 μL was diluted with 495 μL of PBS and used for cell counting with Scepter™ 2.0 Handheld Automated Cell Counter (Merck), whereas the rest was centrifuged for 8 min at 600*g* at 4 °C. The pellet obtained was homogenized in 100 μL of hypotonic lysis buffer, previously described, for each $2{\cdot}10^6$ cells counted. Then, three freezes–thaw cycles in N_2/30 °C were conducted. Subsequently, after centrifugation for 10 min at 12,000*g* at 4 °C, the supernatant was collected, snap frozen in liquid nitrogen and stored at −80 °C until assayed within 21 days.

2.4. Preparation of stock, secondary and working solutions of peptides

For RII peptide, two stock solutions were prepared at 1.00 mM in RNA-free water. One set of stock solutions was used for the preparation of calibrator samples, while the other set was used for quality control (QC) sample preparation. The calibrator set was diluted with reaction buffer in seven working solutions (0.00, 0.12, 1.20, 2.40, 3.60, 4.50 and 6 μM). The other set was diluted to obtain three working QC samples (0.30, 1.50, and 3.90 μM). RIIp and RII-IS peptides stock solutions (1.00 mM) were prepared in RNA-free water. These solutions were diluted with RNA-free water for RIIp, and with EDTA 2 mM for RII-IS to obtain working solutions of 50.0 μM and 2.00 μM, respectively. All solutions were aliquoted and stored at −80 °C until analysis.

2.5. Preparation of calibrators and quality control samples in PBMC

For calibration curve and QC preparation, 40 μL of either working calibration or QC sample, 20 μL of blank PMBC lysates and 60 μL of RII-IS working solution were merged. Final concentrations of 0.0, 0.04, 0.40, 0.80, 1.20, 1.50, 2.00 μM for calibrators and 0.15, 0.50 and 1.30 μM for QC were obtained.

2.6. Calcineurin activity measurement

PMBC lysates (30 μl) were transferred into a tube containing 51 μL of reaction buffer or inhibitor buffer. After vortex and 5 min of incubation at 30 °C, 63 μL of this solution were added into a new tube with 7 μL RIIp (50 μM) to begin the reaction (RIIp, final concentration of 5 μM). After mixing and 15 min of incubation at 30 °C, 60 μL of the reaction tube were added to another tube containing 60 μL of a mixture of 2 mM EDTA and IS working solution (2 μM RII-IS) to stop the reaction. Later, a solid-phase extraction procedure using Oasis® HLB 96 μelution plates (Waters, Milford, USA) was performed to desalt samples. First, well plates were conditioned using 200 μL of methanol and equilibrated with 200 μL of a mixture containing 2% ACN and 0.1% TFA in water (*v/v*). Then, samples were loaded into the plate. Eight hundred μL of a mixture containing 2% ACN and 0.1% TFA (*v/v*) in water followed by 200 μL of water were used to wash the Oasis® plate prior to elution with 60 μL of a mixture containing 80% ACN and 0.1% TFA in water. Finally, eluates were diluted with 120 μL of water and recollection plates were placed in the UHPLC-autosampler at 15 °C.

Quantification of protein on PBMC lysates was made using BCA Reagent Kit (Thermo Fisher). Standard calibrators were prepared diluting BSA in lysis buffer at different concentrations (0.15, 0.45, 0.75, 1.2, 1.8, 2.5 and 3.0 $g{\cdot}L^{-1}$). Moreover, QCs were made to ensure the calibrator curve (0.3, 0.9, and 1.5 $g{\cdot}L^{-1}$). BCA absorbance lecture was made at 540 nm.

2.7. Instrumentation and UHPLC-MS/MS conditions

CNA analysis were conducted using an Acquity®-UPLC® integrated system (Waters). Chromatographic separation was performed in an Acquity®-UPLC® HSS™ T3 C_{18}-column (50 mm × 2.1 mm; 1.8 µm) at 30 °C (Waters). Mobile phase A consisted of 0.1% formic acid (*v/v*) in water, whereas mobile phase B consisted of 0.1% formic acid (*v/v*) in ACN. The mobile phase flow rate was maintained at 0.50 $mL{\cdot}min^{-1}$. From 0.0 to 1.6 min, isocratic conditions were run with 10% of B. Thereafter, from 1.6 to 2.0 min, a column cleaning procedure was performed by increasing non-linearly solvent B to 90%. *Re*-equilibration was performed from 2.0 to 2.5 min at 10% B using a non-linear gradient. The injection volume was 10 µL. Detection was carried out using an Acquity®-TQD® mass spectrometer (MS/MS) (Waters) using electrospray ionization in positive mode. RII and RII-IS peptides were detected in multiple reaction monitoring (MRM) using *mass-to-charge* transitions of 704.9 → 201.2 and 707.4 → 207.3, respectively. The dwell times were set at 0.10 s. The other optimized MS/MS settings were capillary potential 1.0 kV, source temperature 150 °C, desolvation temperature 450 °C, desolvation gas flow rate 900 $L{\cdot}h^{-1}$, cone gas flow rate 50 $L{\cdot}h^{-1}$, collision gas flow 0.20 $mL{\cdot}min^{-1}$ and cone voltage 40 V and collision energy 35 eV for both peptides.

2.8. UHPC-MS/MS method validation

The validation was carried out according to EMA and FDA guidelines [30,31].

2.8.1. Imprecision and bias

For intra- and inter-day imprecision and bias, 6 aliquots of each QC were tested repeatedly in one day and in a single series per day, for 11 non-consecutive days. Imprecision was calculated as coefficient of variation (*CV*) and relative bias (δ_r) as:

$$\delta_r\ (\%) = [(\bar{x}-\mu)/\mu]{\cdot}100$$

where $\bar{x}$ and μ are the mean and the nominal values, respectively. *CV* and δ_r obtained were considered acceptable if they did not exceed 15%.

2.8.2. Lower limit of quantification (LLOQ)

The calibrator 0.04 µM was processed following imprecision and bias study. LLOQ was considered acceptable if the signal-to-noise ratio (S/N) was ≥5, and *CV* and δ_r did not exceed 20%.

2.8.3. Selectivity

An interference study for RIIp was performed in six different batches of PBMC from healthy volunteers. No interference was considered if the peak area response of RIIp peak at the retention time of RII was < 20% of the LLOQ for the analyte, and 5% for the IS.

2.8.4. Carry-over

Carry-over was assessed by injecting three blank calibration samples after the highest calibration sample (2.00 µM). Carry-over was acceptable if the peak area responses in the blank samples were not > 20% of the RII peak area response at the LLOQ, and 5% of the peak area responses of the IS.

2.8.5. Calibration curve

Calibration samples were processed in duplicate once a day. The calibration curves were generated by linear fit (1/X weighting) of the RII/RII-IS area response ratio multiplied by RII-IS concentration (*y*-linear variable) vs. RII concentration (*x*-linear variable). Calibration curves were acceptable if concentrations of the calibration samples were all within ± 15% from the nominal value, except for the LLOQ which ± 20% was allowed.

2.8.6. Recovery

Several RII-spiked PBMC samples were prepared at different concentrations (0.15, 0.50 and 1.30 µM). Recovery was calculated as the mean ratio between the peak area response of six replicates of these samples and the corresponding peak area response of equivalent neat samples. Recoveries were acceptable if their variation among all concentrations was < 15%.

2.8.7. Matrix effect

It was estimated using the ratio of the peak area response in the presence of the matrix (measured by analyzing a blank matrix spiked after extraction with analyte) to the peak area response in the absence of the matrix (pure solution of analyte). IS-normalized matrix factor was calculated by dividing the matrix factor of the RII by the matrix factor of the RII-IS in six different batches of PBMC matrices at 0.15, 0.50 and 1.30 µM of RII. Matrix effect was accepted if its variation among all concentrations was < 15%.

2.9. Application to biological samples

The applicability of this method was evaluated in samples from healthy volunteers ($n = 5$) and renal transplant recipients ($n = 5$) treated with twice daily formulation of TAC after informed consent was accepted. Measurement of TAC concentrations in whole blood was performed using a UHPLC-MS/MS method previously validated [32]. We defined the basal condition as the CNA determined early in the morning for healthy individuals and immediately prior to intake of immunosuppressant drugs for renal patients. Furthermore, to study the effect of CNA during drug exposure time, a PK and PD profiles were performed among 12 h with an intensive sampling (Pre-dose, 1, 3, 6 and 12 h) in renal transplant recipients ($n = 3$).

2.10. Data analysis

CN activity was calculated according to RII formation and normalized by protein quantification of the 20 µl of PBMC lysates and incubation time. The following equation was:

CN activity ($pmol{\cdot}m^{-1}{\cdot}mg\ prot^{-1}$) = [produced RII/(incubation time · protein quantification)].

The comparisons between healthy subjects and renal transplant recipients were assessed by unpaired *t*-test using Prism 6.0 (GraphPad).

3. Results

3.1. Calcineurin activity determination

To quantify phosphatase activity of PBMC extracts, CNA method was performed with the reaction buffer, but also with the inhibition buffer to evaluate background phosphatase activity. Because the amount of RII produced with inhibitor buffer in all the samples was less than our LLOQ, we exclude inhibitor buffer in further analysis. Moreover, from different times proposed in other studies (10, 15, 30 min), we stopped the CN reaction at 15 min as we did not observe enzyme saturation at this point in a lysate concentration between 0.5 and 3 $g{\cdot}L^{-1}$ (data not shown).

RII and RII-IS eluted at ULPC-MS/MS retention time of 0.74 min and the total run time was 2.5 min. Representative MRM chromatograms for the LLOQ (0.04 µM) and for the medium QC sample (0.50 µM) are shown in Fig. 1. For MS parameters, positive ionization showed better sensitivity than negative ionization mode. $[M\text{-}H]^{3+}$ ions were more abundant than $[M\text{-}H]^{2+}$ or $[M\text{-}H]^{4+}$ adducts (Fig. 2A). The selection of

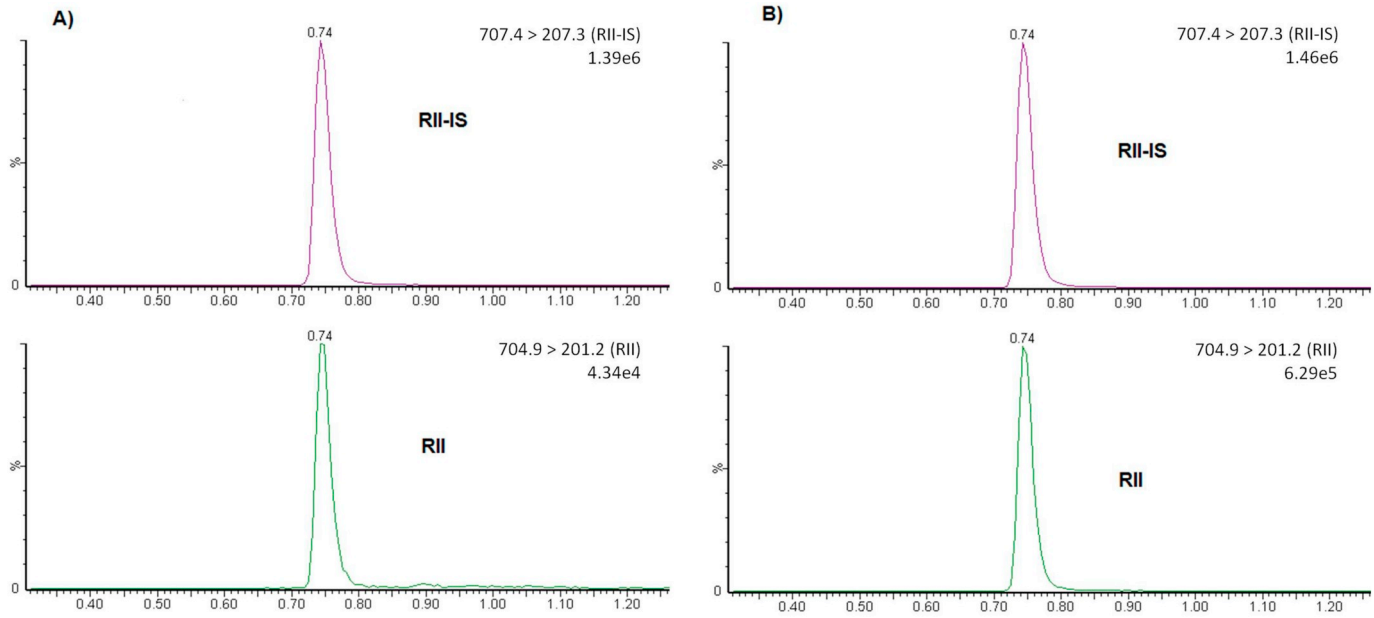

Fig. 1. Representatives MRM chromatograms of RII and RII-IS peptides. (A) For the LLOQ (0.04 µM), and B) for the medium QC sample (0.50 µM).

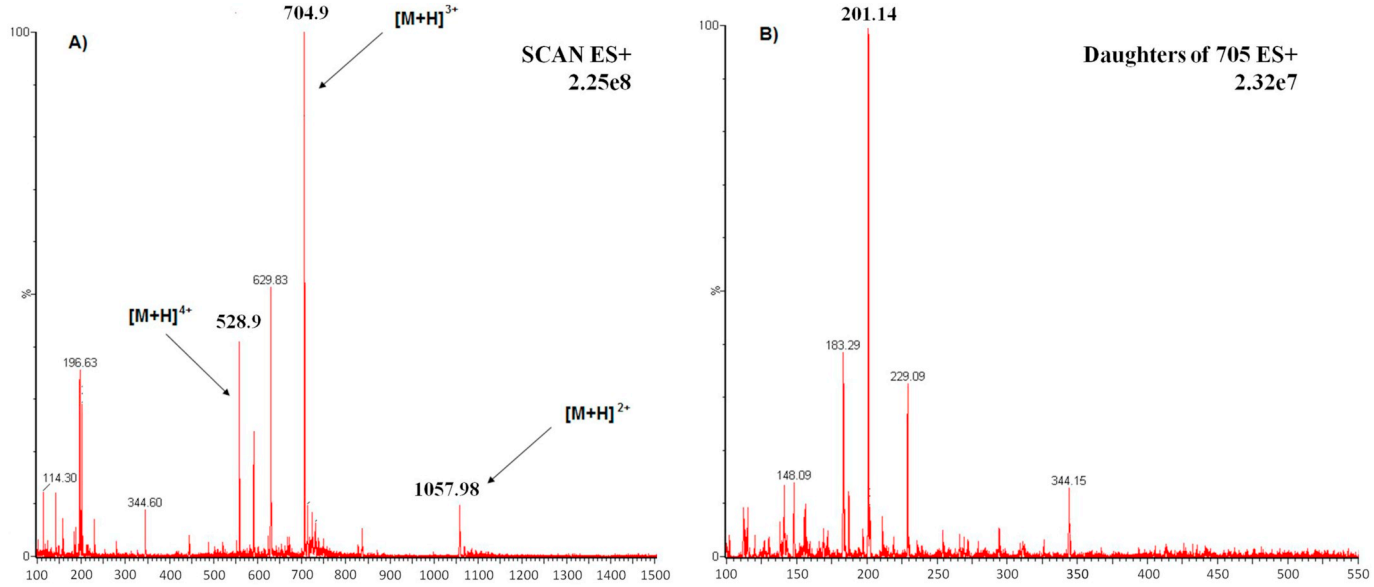

Fig. 2. Parent ion (A) and product ion (B) mass of RII peptide.

the monitored ions after studying the MS/MS fragmentation pattern of RII and RII-IS is illustrated in Fig. 2B. The optimized transitions used for quantification were 704.9 → 201.2 and 707.4 → 207.3 for RII and RII-IS, respectively.

3.2. Validation data

3.2.1. Calibration curve

Calibration curves showed a satisfactory linearity in the range of 0.04–2.00 µM. A characteristic calibration curve equation was $y = 0.33511 \cdot x - 0.00341$ ($r^2 = 0.99664$). The deviations of calculated concentrations from their nominal values ranged from −7.6% to 10.3%.

3.2.2. Imprecision, bias and lower limit of quantification

Data for imprecision and bias are summarized in Table 1. Intra- and inter-day LLOQ were 0.045 µM (S/N = 2464) and 0.043 µM (S/N = 635), respectively.

3.2.3. Selectivity

The peak area responses observed for RIIp in all PBMCs batches at RII retention time were ≤ 3.1% of the LLOQ of RII, being < 0.5% at the RII-IS retention time.

3.2.4. Carry-over

Peak area responses in the three first blank samples processed after the high-concentration sample (2 µM) measurement were 3.1%, 0.8% and 0.1% of the RII peak area response at the LLOQ, respectively. On the other hand, peak area responses were 2.3%, 1.1% and 0.9% of the peak area response of the RII-IS.

3.2.5. Recovery

The recovery at different concentrations for RII at 0.10, 0.50, 1.30 µM were 89.9 ± 3.4%, 91.5 ± 4.2%, 93.3 ± 4.9%, respectively. The recovery for RII-IS at 0.5 µM was 93.6 ± 5.2%.

Table 1
Intra-day and inter-day imprecision and bias values obtained in UPLC-MS/MS for RII substance concentration in PBMCs.

Theoretical concentration (μM)	Intra-day (n = 6)			Inter-day (n = 6)		
	$\bar{x}$ ± s (μM)	CV (%)	δ_r (%)	$\bar{x}$ ± s (μM)	CV (%)	δ_r (%)
0.040 (LLOQ)	0.045 ± 0.001	2.3	11.7	0.043 ± 0.003	7.6	6.3
0.100 (QC1)	0.097 ± 0.002	1.7	−3.2	0.096 ± 0.004	4.0	−4.2
0.500 (QC2)	0.521 ± 0.003	0.5	4.1	0.552 ± 0.024	4.3	10.3
1.300 (QC3)	1.309 ± 0.005	0.4	0.7	1.363 ± 0.042	3.0	4.8

n, number of samples processed; $\bar{x}$, mean values; s, standard deviation; CV, coefficient of variation; δ_r, relative bias; LLOQ, low limit of quantification; QC, internal quality control.

Table 2
Matrix factor and RII-IS-normalized matrix factor for RII substance concentration in PBMCs.

Matrix Lot	Matrix factor (%)			RII-IS-normalized matrix factor (%)		
	0.10 μM	0.50 μM	1.30 μM	0.1 μM	0.50 μM	1.30 μM
1	100.2	95.3	93.5	107.8	102.5	100.6
2	95.4	98.6	98.3	107.4	110.9	110.6
3	92.6	93.3	97.6	111.3	112.1	117.3
4	94.1	93.7	98.6	106.6	106.1	111.6
5	95.7	95.0	99.1	108.4	107.6	112.3
6	100.7	92.9	100.2	121.7	112.3	121.1
$\bar{x}$ (%)	**96.5**	**94.8**	**97.9**	**110.5**	**108.6**	**112.2**
s (%)	**3.3**	**2.1**	**2.3**	**5.7**	**3.9**	**7.0**
CV (%)	**3.4**	**2.2**	**2.4**	**5.2**	**3.6**	**6.2**

$\bar{x}$, mean values; s, standard deviation; CV, coefficient of variation.

3.2.6. Matrix effect

Evaluation of the matrix effect showed ion suppression for RII and RII-IS (Table 2). The mean IS-normalized matrix effect among all concentrations was 110.4 ± 5.5%.

3.3. Clinical application

The mean basal CNA was similar in healthy individuals and renal transplant recipients (426.6 ± 132.3 and 334.4 ± 56.18 pmol·m^{-1}·mg prot^{-1}, respectively) (Fig. 3). Moreover, the extensive PD profiles in renal transplant recipients showed highest inhibition approximately 1 h after drug intake (232.83 ± 13.90 pmol·m^{-1}·mg prot^{-1}). After maximum inhibition, a progressive recovery of CNA to pre-dose levels was observed almost at 6 h after oral administration (pre-dose vs 6 h, 303.58 ± 23.03 vs 277.90 ± 33.28 pmol·m^{-1}·mg prot^{-1}, respectively). In this regard, the highest blood concentration of TAC was obtained at 1 h (18.0 ± 7.8 μg·L^{-1}) decreasing to basal levels approximately 6 h after drug intake (pre-dose vs 6 h, 7.9 ± 2.7 vs 9.5 ± 1.3 μg·L^{-1}, respectively). These profiles showed that the maximum inhibition of CNA was paralleling the highest concentration of TAC in whole blood (Fig. 4).

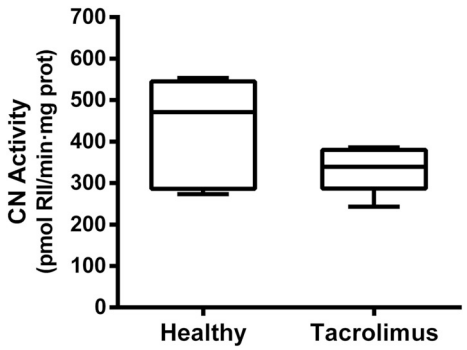

Fig. 3. Comparison of CNA between healthy volunteers and renal transplant patients receiving TAC-twice-a-day formulation at pre-dose time (n = 5).

4. Discussion

Little is known about the PD monitoring of TAC but the methodology to optimize TAC exposure and efficacy is promising. In this study, a new PD technique based in UHPLC-MS/MS was developed and validated for the measurement of CNA in PBMC to address the potential relationship between drug exposure and PD overtime in renal transplant patients.

This assay is based on the quantification of dephosphorylated peptide as a product of CNA. To obtain easy and feasible results in a single tube assay, we used the inhibitor cocktail for phosphatases only described by Carr et al. [29]. RIIp peptide does not have a specific sequence only for CN dephosphorylation and could be dephosphorylated by other families of phosphatases. Thus, the combination of sanguinarine chloride and okadaic acid without $MgCl_2$ was chosen as the best option to obtain a good inhibition of PP1 and 2A, C phosphatases family. As it has been described by other authors, we also excluded EDTA and EGTA from lysis or reaction buffer to avoid calcium chelators that could interfere with CNA [10,25,29]. Furthermore, we attempted to

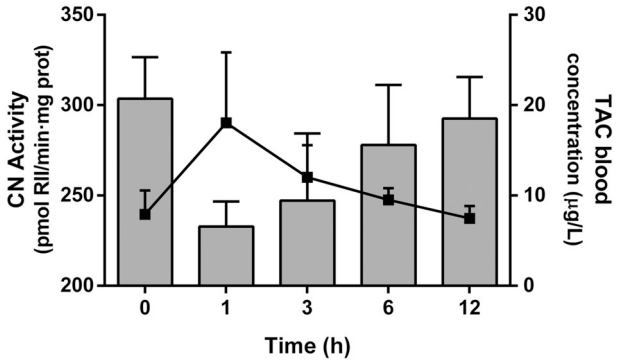

Fig. 4. Pharmacodynamic and pharmacokinetic monitoring of three kidney transplant recipients receiving twice daily formulation of TAC at 5 different timepoints (0, 1, 3, 6, 12 h).

maintain the ratio of TAC on the different subsets of whole blood. Bearing in mind that TAC is a drug that preferentially binds to red blood cells [33], we removed them from our lysate to avoid not intra-lymphocytic TAC to the final lysate that could alter phosphatase activity [10,34]. Moreover, to preserve intracellular TAC during the PBMC isolation, we chose the use of low temperatures (4–10 °C) to inhibit drug extrusion from P-glycoprotein [34–36].

Concerning the chromatographic conditions, various combinations of mobile phases and gradient elution modes were performed to achieve a good resolution and symmetric peaks, shorter retention time and better peak shape. The combination of water and ACN with formic acid as mobile phase and the shorter chromatographic column enabled a shorter run chromatographic time and faster determination [26,29]. Relating the internal standard (IS), stable leucine labeled RII peptide for RII was used as a first choice for the analysis of the CNA [29].

Regarding of analytical performance characteristics, the CV and δ_r obtained did not exceed the EMA criteria (15%) [30], indicating that the proposed UHPLC-MS/MS method provides acceptable precision and trueness, improving on other methods reported [26–29]. Moreover, the use of UHPLC-MS/MS reached lower LLOQ (0.04 μM) compared to other techniques [10,26,29]. This LLOQ enabled us to measure the RII values in TAC PBMCs, difficult to analyze using other methods. Furthermore, no significant interfering peaks were present in any sample assayed indicating an acceptable selectivity. Also, the UPLC–MS/MS method showed acceptable linearity between 0.04 and 2.00 μM. According to our results, recoveries were considered constant and reproducible. Carry-over and matrix effect have not been studied before and are crucial for data analysis [10,25–29]. Our results presented a negligible carry-over and an acceptable matrix effect which is corrected by RII-IS.

Determination of CNA assay was tested in healthy volunteers and transplant patients receiving TAC immunosuppression. In contrast to other studies that presented differences in both groups [26,27], similar levels of CNA were obtained between healthy volunteers and kidney transplant patients at pre-dose time (426.6 ± 132.3 and 334.4 ± 56.18 $pmol \cdot m^{-1} \cdot mg\ prot^{-1}$). This could be explained by the high variability of CNA, especially in healthy volunteers whereas the CNA in TAC-treated patients was more constant. Similar results have been observed in renal transplantation with long term TAC treatment [16,29].

This sensitive technique enables us to analyze slight differences in CNA during drug dose intervals of TAC. Analyzing the PD profile of TAC-twice-a-day formulation, we could observe a maximum inhibition of CNA at 1 h after drug intake when blood concentration reached the highest value [9,14,16,17]. Once maximum inhibition was reached, CNA values returned progressively almost to the minimum inhibition baseline pre-dose after 12 h of follow-up.

In conclusion, an easy UHPLC-MS/MS method was developed and validated following international recommendations [30,31] for measurement of CNA in PBMCs. With regard to PBMCs are the biological matrix for immunosuppressor drugs, the measure of CNA on these cells would supply clinical relevance in comparison to other common techniques. In comparison with Carr et al [29] our method improves the extraction step by the lysis of red blood cells and the management at low temperatures that allowed us to measure CNA more specifically maintaining intra-lymphocytic source of TAC [34–36]. Moreover, our UPLC-MS/MS method improves the sensibility achieving a LLOQ lower in front of other techniques. Considering the shorter time of analysis and analytical performance characteristics obtained, this method would be suitable for entering in the clinical setting than the other techniques. PD monitoring could be a useful tool to refine TAC-based immunosuppression in transplantation in terms of TAC efficacy and safety. This methodology may encourage to perform PD monitoring in a long prospective cohort of patients.

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