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UPLC–MS/MS assay validation for tacrolimus quantitative determination in peripheral blood T CD4+ and B CD19+ lymphocytes

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ABSTRACT

Monitoring tacrolimus (Tac) exposure in cell matrices enriched with lymphocytes can improve Tac therapeutic drug monitoring (TDM) in solid organ transplant recipients. An UPLC–MS/MS based assay for Tac quantification in peripheral blood T CD4+ and B CD19+ lymphocytes was developed. Peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation and highly purified (purity >90%) T CD4+ and B CD19+ cell suspensions were acquired by magnetic negative selection from whole blood of 6 healthy volunteers. The purity of lymphocyte suspensions was checked by flow cytometry. Tac extraction was performed in a liquid–liquid zinc sulfate, methanol and acetonitrile based medium. Ascomycin was used as internal standard. The equipment used was a Waters® Acquity™ UPLC system (Waters Corporation, Milford, MA, USA). The chromatographic run was performed on a Waters® MassTrak TDM C18 (2.1 × 10 mm) column (Waters Corporation, Milford, MA, USA). at a flow rate of 0.4 mL/min. The instrument was set in electrospray positive ionization mode. The method was validated according to Clinical Laboratory Standard Institute (CLSI) guidelines and showed a high sensitivity and specificity over a range of 0–5.2 ng/mL in PBMC, 0–5.0 ng/mL in T CD4+ Lymphocytes and 0–5.3 ng/mL in B CD19+ lymphocytes. Precision was appropriate with CV of intra-assay quantifications ranging from 4.9 to 7.4%, and of inter-assay quantifications from 7.2 to 13.9%. Limit of detection and quantification were 0.100 and 0.115 ng/mL in PBMC, 0.058 and 0.109 ng/mL in T CD4+ and 0.017 and 0.150 ng/mL in B CD19+ cells. Matrix effect was not significant among all the studied matrices. Samples showed stability for Tac quantification over a period of 90 days either at room temperature or at −30 °C storage conditions. The method was applied to clinical samples of 20 kidney transplant recipients. Concentrations ranged from 2.200 to 11.900 ng/mL in whole blood, from 0.005 to 0.570 ng/10^6 cells in PBMC, from 0.081 to 1.432 ng/10^6 cells in T CD4+, and from 0.197 to 1.564 ng/10^6 cells in B CD19+ cell matrices. The method has potential applicability for Tac TDM in solid organ transplant recipients.

1. Introduction

Tacrolimus (Tac) is the core immunosuppressant used in modern clinical solid organ transplantation to prevent allograft rejection. Due to its narrow therapeutic index and significant intra- and inter-patient variability, therapeutic drug monitoring (TDM) is recommended to improve efficacy and to limit side effects[1].

Although monitoring tacrolimus in whole blood is near universal among transplant centers, the incidence of acute rejection in the first year after kidney transplantation remains significant. To date, the optimal blood concentration of tacrolimus able to predict the risk of rejection after kidney transplantation remains poorly defined [2] with some patients presenting rejection although their immunosuppressive drugs are within the blood therapeutic window. It is possible that the variability of the *p*-glycoprotein efflux drug transporter keeps the Tac concentration low within the lym-

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phocytes although the blood concentration is considered adequate. This has already been shown with Tac [3] and cyclosporine A [4], another calcineurin inhibitor.

The mechanism of action of Tac mainly consists in blocking calcineurin/NFATC dependent activation pathway in lymphocytes [5]. Monitoring tacrolimus concentration in cellular matrices enriched with lymphocytes has recently emerged as alternatives to improve therapeutic tacrolimus monitoring [6]. A poor correlation between whole blood and PBMC concentrations of tacrolimus has been reported [6,7]. Tac concentrations in PBMC was shown to be lower among liver transplant recipients with acute rejection [8].

However, no information is available regarding Tac distribution in specific lymphocyte subsets. This information would be important in patients who present acute rejection with drugs within the therapeutic window.

The objective of this study is to present a reliable and validated method of Tac quantification in human peripheral blood T CD4+ and B CD19+ lymphocytes.

2. Methods

2.1. Study design and population

This is a bioanalytical method validation study designed to provide information about the application of ultrahigh performance liquid chromatography (UPLC) combined mass spectrometry (MS) for quantitative determination of TAC in peripheral blood mononuclear cells (PBMC), T CD4+ and B cell suspension matrices. Six healthy subjects and 20 stable kidney transplant recipients were enrolled at Hospital das Clínicas de Sao Paulo, University of Sao Paulo School of Medicine, Brazil, from March 2016 to December 2016. Self-reports of health were used to assess the health condition of healthy volunteers and to confirm they were free of any medications intake for at least 6 month before inclusion. Kidney transplant recipients had stable graft function with no prior rejection episodes and under tacrolimus (Generic Tac, Tarfic®, Libbs Farmaceutica, Sao Paulo, SP, Brazil), mycophenolate sodium (Myfortic®, Novartis, Basel, Switzerland) and prednisone maintenance immunosuppressive therapy. The institutional board of ethics in research approved the study protocol (CAAE–41558915.9.0000.0068). Healthy volunteers and patients provided formal informed consent prior to the study enrollment.

2.2. Blood collection and cell preparation

A sample of 40 mL of heparinized whole blood was collected from healthy subjects and patients at study recruitment. Simultaneously, an additional sample of 4 mL of whole blood in EDTA containing tube was collected from patients for Tac quantification in whole blood. Blood samples from patients were collected after an overnight fasting of 12 h post TAC oral dose. Total leukocyte and lymphocyte counts were performed according to standard clinical laboratory methods. PBMC were separated by standard Ficoll (GE Healthcare, Uppsala, Sweden) density gradient centrifugation. In brief, 40 mL of whole blood were diluted with 40 mL of NaCl 0.9% solution (1:2 blood mixture of 80 mL). Aliquots of 35 mL of the blood mixture were carefully added in 50 mL polypropylene tubes each containing 12.5 mL of Ficoll solution without mixing. After centrifugation at 650*g* for 35 min at room temperature, the PBMC layers obtained in the tubes were collected and pooled in a 50 mL polypropylene tube. The cells were then washed with 50 mL of NaCl 0.9% solution at 4 °C (centrifugation at 900*g* for 10 min). Finally, the pellet was suspended with 10 mL of NaCl 0.9% solution at 4 °C. Ten microliters of cell suspension was removed for cell counting. An aliquot of 10^6 harvested cells were washed again, resuspended with 1 mL of Dulbecco's Phosphate Buffered Saline (DPBS, GIBCO, Carlsbad, CA, EUA) and stored at −80 °C until analysis.

Purified T CD4+ and B lymphocytes suspensions were obtained by magnetic negative selection using human CD4+ T cell isolation kit and human B cell isolation kit II (both Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), respectively. After centrifugation at 300*g* for 10 min, the PBMC pellet was resuspended in 80 μL of buffer solution (PBS, 1% FCS and 2 mM EDTA) per 10^7 cells. Than 20 μL of the cocktail of biotinylated antibodies were added per 10^7 cells in the suspension. After an incubation time of 5 min at 4 °C, 60 μL of buffer solution per 10^7 cells and 40 μL of MicroBead cocktail were added to the suspension. After an incubation time of 5 min at 4 °C, the cell suspension was applied to the column. The full process of cell surface labeling and column separation was repeated again to increase cell yields and purity. All procedures were performed at 4 °C to minimize TAC efflux from cells.

2.3. Flow cytometry analysis of purity of lymphocyte suspensions

Purity of the PBMC, T CD4+ and B lymphocyte suspensions were analyzed immediately after cell preparation by flow cytometry. Aliquots of cell suspensions were separated, washed and then surface stained with titrated amounts of the following mouse anti-human monoclonal antibodies: anti-CD3-FITC (SK7), anti-CD8-PE (SK1), anti-CD45-PerCP (2D1 (HLe-1), anti-CD4-APC (SK3) (BD Multitest™ CD3/CD8/CD45/CD4) and anti-CD19-APC-Cy7 (SJ25C1). Live cells were discriminated using BD Horizon™ Fixable Viablity Stain (FVS 450). All these antibodies and reagent were from BD biosciences (San Jose, CA, USA). Flow cytometry was performed in a FACSCanto II (BD Biosciences) cytometer equipped with the acquisition software BD FACSDiva (Becton Dickinson®). FlowJo 9.1 software (TreeStar Inc, San Carlos, CA, USA) was used for data analysis. The gating strategies used to check the purity of cells suspension are shown in Fig. 1. Only samples with ≥ 90% of purity of the lymphocyte subsets of interest were further processed for method validation and TAC quantification. The methodology to obtain such a high concentration of specific cells is being reported in another paper.

2.4. Preparation of standard and quality control samples in three different matrices

Stock solution of Tac (Libbs® Farmaceutica, Sao Paulo, SP, Brazil) was prepared by dissolving lyophilized Tac with DMSO (Sigma-Aldrich Inc, Missouri, USA) to obtain a 1.0 mg/mL solution. A working solution of 1000 ng/mL was obtained by diluting the primary stock solution with methanol (Sigma-Aldrich Inc, Missouri, USA): water. Both solutions were stored at −80 °C. Aliquots containing 10^6 cells suspended in DPBS were used to prepare standard and quality control (QC) samples separately with the three different cell matrices. Tac standard samples were prepared by dilutions from the working solution in blank PBMC, T CD4+ and B lymphocytes to achieve working solutions of 10 ng/mL, and thereafter by successive dilutions to achieve a concentration interval ranging curve from 0 to 5.0 ng/mL in the specific matrices. Two QC samples were prepared with each one of the cell matrices with concentrations of 0.100 and 0.800 ng/mL for PBMC, 0.057 and 0.210 ng/mL for T CD4+ lymphocytes and 0.020 and 0.250 ng/mL for B CD19+ lymphocytes. We also prepared additional QC samples by pooling cell suspensions of T CD4+ and B CD19+ lymphocytes obtained from patient samples. These pooled samples were tested in replicates of 20 and the mean obtained concentrations were 0.262 ± 0.07 ng/mL (CV 2.7%) and 0.322 ± 0.03 ng/mL (CV 1.0%) ng/mL for T CD4+ lymphocytes and 0.103 ± 0.002 ng/mL (CV 2.0%) and 0.097 ± 0.003 ng/mL