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A high performance liquid chromatography tandem mass spectrometry for the quantification of tacrolimus in human bile in liver transplant recipients

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ABSTRACT

Tacrolimus whole-blood concentrations imperfectly reflect concentrations at the effect site. Tacrolimus concentrations in the transplanted organ could be more relevant to predict rejection events. Because liver biopsy cannot be repeatedly performed after liver transplantation, we suggested measuring tacrolimus in the bile to have a cost-effective and clinically implementable surrogate marker of intra-hepatic tacrolimus concentration. We developed and fully validated a liquid chromatography–tandem mass spectrometry method for the determination of tacrolimus in human bile. Sample purification was achieved using protein precipitation and liquid–liquid extraction with ethyl-acetate. Gradient elution was performed using a C18 analytical column with a 5 min run-time. The method was linear from 0.5 ng/mL to 20 ng/mL. In this concentration range, within-day and between-day precisions as well as overall bias were within ±15%. Matrix effect was fully corrected by the internal standard (ascomycin). The assay was optimized to achieve good selectivity in this complex biological matrix. Tacrolimus was found to be stable in bile stored 6 months at −80 °C, after 3 freeze and thaw cycles, 20 h at room temperature and 24 h in extracts kept at 15 °C in the auto-sampler. The method was applied to quantify tacrolimus in bile from liver transplant recipients. It allowed getting preliminary data about tacrolimus excretion profile in bile and showed the lack of correlation between tacrolimus whole blood concentration and tacrolimus liver exposition. This alternative and innovative analytical approach of tacrolimus bio-analysis appears suitable for further studies evaluating relevance of biliary tacrolimus concentration as a new pharmacological marker of immunosuppressive activity.

1. Introduction

Long-term survival following liver transplantation has slowly evolved during the last five years. The rate of acute graft rejection (ACR) also remained stable with a frequency of about 8–15% at one year despite effective immunosuppression [1]. Cornerstone of allograft rejection prevention, tacrolimus prevents the immune response against the graft by inhibiting interleukin-2 production by lymphocyte T cells [2]. The drug presents highly variable pharmacokinetics and narrow therapeutic range making challenging the achievement of the balance between therapeutic efficacy and occurrence of side effects [3]. Therapeutic drug monitoring (TDM) of tacrolimus levels in whole-blood contributed to decrease the risk of ACR as well as the onset of adverse reaction [4]. However, some patients exhibit ACR while having blood levels within the recommended therapeutic range. This suggests that tacrolimus trough whole-blood concentrations imperfectly reflect tacrolimus concentrations at the effect site. Thus, looking for novel approaches of immunosuppressive drugs monitoring is needed to better pre-

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vent ACR. In a prospective study in liver transplant recipients, Capron et al. [5] demonstrated the lack of correlation between tacrolimus trough whole-blood concentrations and the score of graft rejection, whereas tacrolimus intra-hepatic and peripheral blood mononuclear cells (PBMC) concentrations correlated well with incidence and intensity of histologically proved ACR. This work suggested that measuring tacrolimus concentrations in liver biopsy or in PBMC could better predict clinical outcomes. The relevance of the determination of immunosuppressive drug concentration in the transplanted organ was also highlighted by Staatz et al. [6]. However, such approaches of tacrolimus quantification are either invasive or expensive and the analytical workflow might be difficult to implement in daily clinical and laboratory practice [4]. We hypothesized that tacrolimus concentration in bile could be a surrogate marker of tacrolimus intra-hepatic concentration. The measurement of tacrolimus in bile appears cost-effective and suitable with clinical practice when T-tube biliary drainage is inserted in patients during surgery. Tacrolimus biliary concentration could reflect the free and non-metabolized fraction of the drug available at the target organ which is responsible for its pharmacological effect. Besides, tacrolimus is extensively metabolized by the liver and biliary excretion is thought to be the major route of elimination of its metabolites [7]. Polymorphisms in genes involved in this metabolism are a major cause of treatment response variability between patients [6]. Thus, tacrolimus concentration in bile could directly depend on the magnitude of drug metabolism, leading to provide valuable information about its pharmacokinetics. Studies reporting tacrolimus determination in bile are sparse and were published a few decades ago [8–10]. They are mainly focused on investigating the drug metabolic pathway without any extensive description of the analytical validation which is unfortunate because this matrix is known to be associated with several bioanalytical challenges [11]. Bile is an unconventional alternative matrix for TDM and demonstration of the reliability of the analysis is a critical step before any clinical application. Thus, prior to investigating whether biliary tacrolimus concentration could be a good marker of its immunosuppressive activity, we aimed at developing and validating, according to international guidelines [12], a high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) method for the determination of tacrolimus in bile from liver transplant recipients. The analytical method was then applied to analyze bile samples from liver transplant recipients.

2. Material and methods

2.1. Chemicals and material

Tacrolimus was purchased from LGC standards (Folsheim, France). Ascomycin was purchased from Sigma-Aldrich (Saint-Quentin, France). Methanol and acetonitrile of LC/MS grade were purchased from Carlo Erba Reagents (Val de Reuil, France). Ethyl acetate, methyl-*ter*-butyl ether (MTBE) and dichloromethane were obtained from VWR international (Fontenay sous Bois, France). Ammonium acetate, zinc sulphate heptahydrate and formic acid were obtained from Fisher Chemicals (Waltham, USA). Water was purified using a Milli-Q® Ultrapure Water System (Merck Millipore, Milford, MA, USA). Beta-glucuronidase enzymes from E. Coli (type IX-A), Helix pomatia and bovine liver origins were purchased from Sigma-Aldrich (Saint-Quentin, France). OASIS® HLB SPE 96-wells plate (30 mg) and OSTRO™ 96-wells plates were purchased from Waters (Saint-Quentin, France).

Human bile was collected from the T-tube biliary drainage inserted in biliary anastomosis during the surgical procedure. Drug free human bile was collected before the onset of the immunosuppressive treatment. Drug-free bile samples from 7 individual liver transplant recipients were used to prepare a batch of pooled biles used in the validation and the clinical application analysis.

2.2. HPLC–MS/MS equipment and conditions

The chromatographic system (ThermoFisher, San Jose, CA, USA) included a Surveyor Autosampler and an HPLC Surveyor MS pump. A C18 Hypersil Gold column (50 × 2.1 mm, 3 μm) fitted with a guard column (10 × 2.1 mm, 3 μm) (Thermo Electron, Dreieich, Germany) was used and maintained at a constant temperature of 60 °C. The autosampler was set at 15 °C during analysis. To measure tacrolimus in bile, gradient elution was performed with a mobile phase composed of a mixture of 0.1% formic acid and 2 mM ammonium acetate in water (eluent A) or in acetonitrile (eluent B). A multistep gradient adapted from Dubbelboer et al. [13] was used at a flow rate of 0.4 mL/min and was programmed as follows: equilibration at initial conditions with 40% of B, increase to 70% of B from 0 min to 0.1 min, increase to 75% of B from 0.1 min to 1.4 min, increase to 100% of B from 1.8 min to 2.45 min, stabilization at 100% of B from 2.45 min to 3.10 min, reversion to the starting conditions at 40% of B from 3.1 min to 3.15 min and re-equilibration with the initial composition from 3.15 min to 5.0 min. The total run time was 5.0 min.

The HPLC system was interfaced with a triple stage quadrupole mass spectrometer Finnigan™ TSQ® Quantum Discovery Max (ThermoFisher, 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 10 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) and 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 acquisition and analysis were performed using LCQuan software, version 2.5.6 (ThermoFisher, San Jose, CA, USA).

2.3. Preparation of standards (Std) and quality control (QC) samples

Two batches of stock solutions of tacrolimus were independently prepared in methanol at a concentration of 1000 ng/mL (one for QC and one for Std). A stock solution of ascomycin was prepared in acetonitrile at a concentration of 500 ng/mL. These stock solutions were stored at −80 °C. Working solutions of each calibration Std and each QC level were daily prepared by dilution of the tacrolimus stock solution in methanol. A working solution of ascomycin used as internal standard (IS) was daily prepared in acetonitrile at the concentration of 250 ng/mL.

Aliquots of 250 μL of drug-free human bile were spiked with corresponding working solutions to obtain 6 non zero calibrators levels at 0.5, 1, 1.5, 5, 10, 20 ng/mL and QC samples at 0.5 (Lower limit of quantification), 1.5 (Low), 10 (Medium) and 20 ng/mL (High). The percentage of organic solvent in bile was lower than 8%.

2.4. Sample preparation

An aliquot of 250 μL of bile was transferred into a 4 mL glass tube. IS was added (40 μL of ascomycin 250 ng/mL) and the sample was diluted with 800 μL of water and vortexed 3s. Then, precipitation was performed by adding 300 μL of a mixture of acetonitrile/zinc sulphate 0.05 M in water 1:1 v/v followed by a liquid/liquid extraction using 2 mL of ethyl acetate. Samples were gently mixed for 10 min and centrifuged at 3000*g* for 10 min at 4 °C. The supernatant was evaporated to dryness under nitrogen flow at room temperature. The sample was reconstituted with 500 μL of