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Quantification of cyclosporine A in peripheral blood mononuclear cells by liquid chromatography-electrospray mass spectrometry using a column-switching approach

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Abstract

As a potential alternative to cyclosporine A (CsA) monitoring in whole blood, a sensitive and selective method was developed for quantifying this immunosuppressive drug in human peripheral blood mononuclear cells (PBMCs) by liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS). PBMCs were isolated from whole blood by density gradient centrifugation. After purification, cell counts were performed to express CsA amounts per single cell. The pelleted cells were then lysed and CsA was extracted with methanol (MeOH) containing 27-demethoxy-sirolimus as internal standard. After evaporation of the supernatant under nitrogen, the residue was reconstituted in MeOH, further diluted with water and injected onto a column-switching unit. On-line solid-phase extraction was performed using a C8 column with an acidic aqueous mobile phase containing 5% MeOH. The analytes were transferred in the back-flush mode on a C18 column with 65% MeOH and the chromatographic separation performed with a MeOH gradient (65–90%). The detection was carried out with a single quadrupole analyzer and the sodium adducts $[M + Na]^+$ were monitored for quantification. This sensitive method was fully validated in the range of 5–400 ng/mL. This allowed the measurement of very small CsA amounts present in cells up to 0.5 fg/PBMC in clinical samples. Trueness (95.0–113.2%), repeatability (5.1–9.9%) and intermediate precision (7.0–14.7%) were found to be satisfactory. This method represents a new potential tool for therapeutic drug monitoring of CsA and could be used in clinical conditions if the utility of intracellular measurements is confirmed in prospective clinical trials.

Keywords: Cyclosporine A; PBMCs; Intracellular; LC–MS; Column-switching; TDM

1. Introduction

Cyclosporine A (CsA), a highly lipophilic cyclic undecapeptide (Fig. 1), is a commonly used immunosuppressive drug in organ transplantation [1]. It binds to cyclophylin, a cytoplasmic receptor present in T lymphocytes, resulting in an inhibition of calcineurin, a key enzyme in the intracellular signaling pathway activated after antigenic stimulation. CsA leads to a large decrease in cytokine production, resulting in an inhibition of T lymphocytes activation and proliferation [2]. CsA exhibits a high degree of pharmacokinetic variability, notably due to its metabolism by cytochrome P450 3A4/5 enzymes [3] and transport by the drug efflux transporter P-glycoprotein, encoded by the human *ABCB1* gene [4]. Furthermore, CsA has a narrow therapeutic index and is subject to many drug–drug interactions [5], therefore therapeutic drug monitoring (TDM) of this agent is usually performed [6].

In whole blood, CsA is distributed in erythrocytes (41–58%), lymphocytes (4–9%), granulocytes (5–12%) and plasma (32–47%) [7]. It is currently recommended to measure CsA levels in whole blood [8,9], because the erythrocyte-to-plasma distribution ratio is greatly variable and principally depends on drug concentration, lipoprotein levels, hematocrit and

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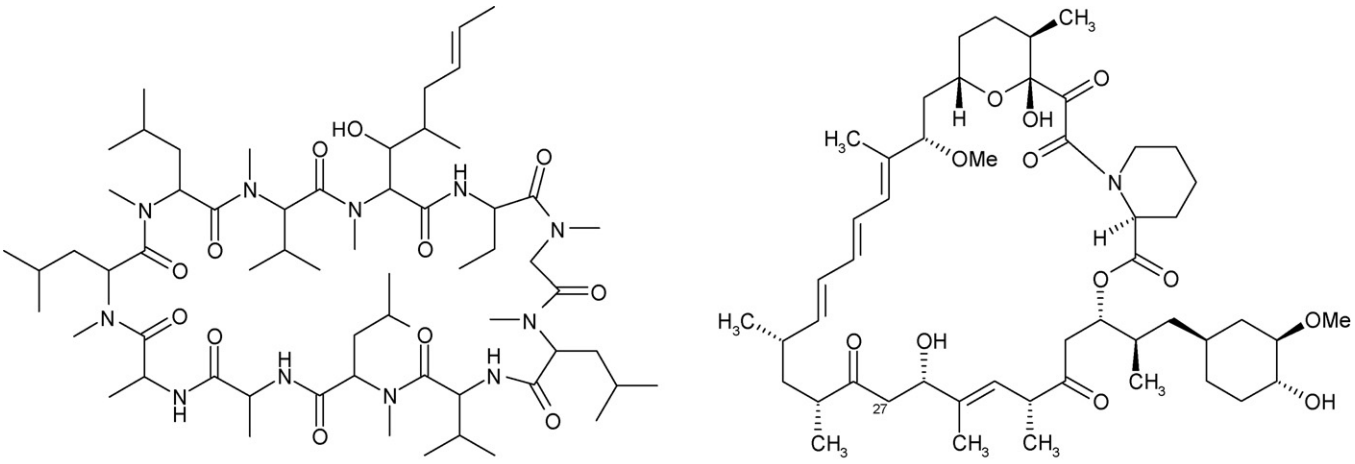

Fig. 1. Chemical structures of the studied compounds.

temperature [10–12]. For many years, the standard in the TDM of CsA was to measure trough concentration (C_0), but more recent findings have shown that CsA quantification at 2 h post-dosing (C_2) was a better predictor of the clinical outcome [13,14]. Immunoassays are largely used for the quantification of this drug [15], but despite the selectivity of these assays, cross-reactivity with metabolites can still occur, resulting in an overestimation of the concentrations [16–18]. Therefore, it is preferable to use a separative method for the quantification of CsA. Several approaches using LC–UV, LC–MS or LC–MS/MS have been developed [19–26].

CsA is removed from the lymphocytes by P-glycoprotein transporter present in the membrane of these cells [27]. The expression and activity of this protein is variable between individuals due to genetic (*ABCB1* gene polymorphisms) and environmental (xenobiotic) factors [28]. This active transporter might influence CsA levels in the target compartment. Masri et al. have proposed to measure CsA directly in human peripheral blood mononuclear cells (PBMCs) using an immunoassay [29]. Interestingly, no correlation between whole blood and intracellular CsA levels was obtained. Furthermore, lower intracellular drug amounts have been observed in patients with acute rejection, however, no differences were seen between patients with or without rejection in whole blood concentrations [29–33]. Intracellular CsA concentration measurements could correlate better with clinical events than in whole blood and offer an attractive perspective in TDM of this drug.

In the present work, a validated selective and sensitive analytical method for the quantification of CsA in PBMCs by LC–MS is proposed. PBMC extracts were purified by on-line solid-phase extraction to obtain maximum selectivity towards endogenous compounds. The method was fully validated including function response estimation, limit of quantification (LOQ), trueness, repeatability and intermediate precision.

2. Experimental

2.1. Chemicals, biologicals and material

CsA, formic acid and sodium formate were purchased from Fluka Chemie (Buchs, Switzerland) and 27-demethoxy-sirolimus was a kind gift from Wyeth-Ayerst Research (Princeton, USA). All reagents and solvents were of analytical grade. Methanol (MeOH) was obtained from Biosolve Ltd. (Valkenswaard, The Netherlands). Ultra-pure water was supplied by a Milli-Q Water Purification System from Millipore (Molsheim, France). Phosphate-buffered saline (PBS) GIBCO^{TM} solution was obtained from Invitrogen (Grand Island, USA) and Ficoll-Paque^{TM} Plus solution from Amersham Biosciences AB (Uppsaia, Sweden). Blank PBMCs were isolated from buffy coat (leukocyte concentrates containing plasma and little contamination with erythrocytes and platelets) obtained from the Blood Transfusion Centre of the University Hospitals of Geneva. Blood samples were obtained from healthy volunteers that participated to a clinical research protocol approved by the local Ethic Committee (University Hospitals of Geneva). The volunteers received a single oral dose (2 mg/kg) of CsA. BD Vacutainer® CPT^{TM} tubes used for the isolation of PBMCs from whole blood were purchased from Becton Dickinson (Franklin Lakes, USA).

2.2. Instrumentation

PBMC counts were performed on a Sysmex® XE-2100 instrument (Sysmex Corporation, Kobe, Japan). Quantification of CsA was performed by LC–MS with a column-switching system (Fig. 2), consisting of an Agilent Series 1100 LC system (Agilent Technologies, Palo Alto, USA) including an autosampler, binary pump (pump 2) and six-port switching valve, and an additional Agilent Series 1050 LC pump (pump 1). Instrument control and data acquisition were processed by the ChemStation Software, Revision B.01.01 (Agilent Technologies). The