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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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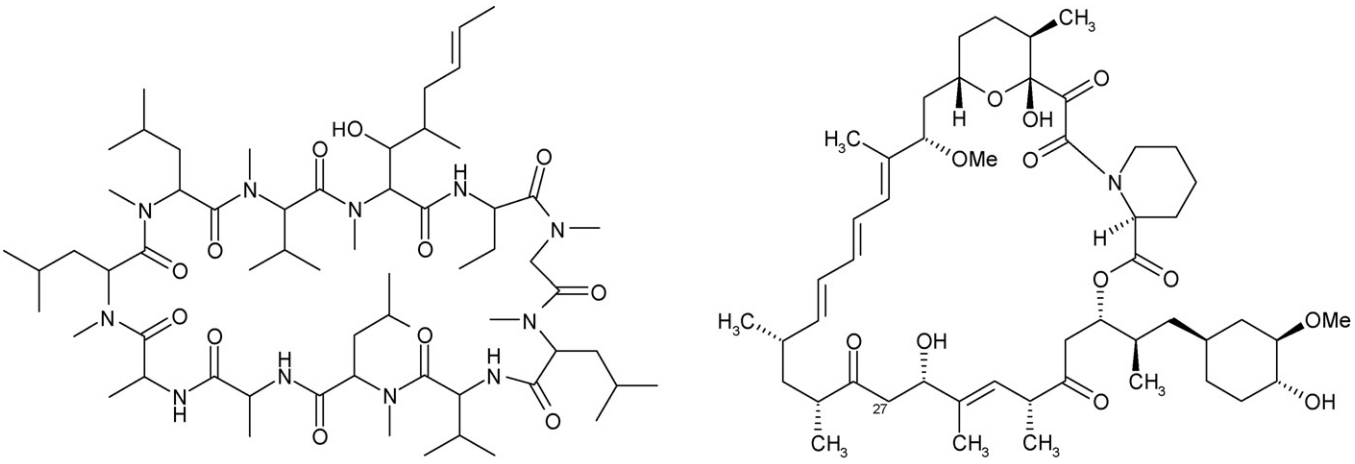

Fig. 1. Chemical structures of the studied compounds.

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

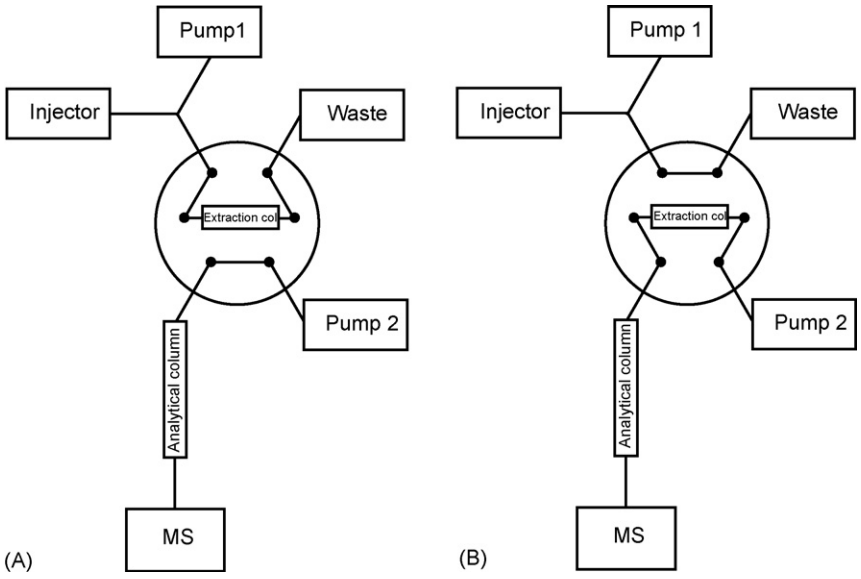

Fig. 2. Column-switching system. (A) System configuration for steps 1 (loading) and 3 (analysis). (B) System configuration for step 2 (transfer).

on-line solid-phase extraction was performed on a XTerra™ MS C8, 5 μm, 2.1 × 10 mm (Waters® Corporation, Milford, USA) at 40 °C and the separation of the analytes on a XTerra™ MS C18, 5 μm, 2.1 × 50 mm at 50 °C fitted with a XTerra™ MS C18, 5 μm, 2.1 × 10 mm pre-column. An in-line filter was placed prior to the extraction column. The LC system was coupled to an Agilent Series 1100 UV-detector or an Agilent Series 1100 MSD single quadrupole MS equipped with an orthogonal electrospray ionization (ESI) interface.

2.3. Sample preparation and LC analysis

2.3.1. Isolation and purification of PBMCs

PBMCs were isolated from whole blood by density gradient centrifugation. Peripheral venous blood (approximately 8 mL) was collected into BD Vacutainer® CPT™ tubes and centrifuged at 1800 × *g* for 30 min at 4 °C. The PBMC layers were collected and washed with 10 mL of PBS at 4 °C. After centrifugation at 300 g for 15 min at 4 °C, the supernatants were discarded. The cell pellets were placed in suspension with 2.5 mL of PBS at 4 °C. Two 250 μL aliquots were taken for cell counting. PBS (10 mL) was added to the remaining cell suspensions. After centrifugation at 300 × g (15 min at 4 °C), the pellets were stored at −80 °C until LC–MS analysis.

2.3.2. Cells lysis and CsA extraction

MeOH (1400 μL) containing 27-demethoxy-sirolimus as internal standard (I.S.) at 10 ng/mL was added to the pellets of PBMCs. The mixtures were vortexed for 2 h at room temperature (RT) allowing cells lysis and CsA extraction. After centrifugation at 2300 × *g* for 5 min, the supernatants were collected and evaporated under nitrogen. The residues were reconstituted with 140 μL of MeOH (vortexed for 15 min) and 50 μL were transferred into vials for the LC–MS analysis. Samples were diluted with 33 μL of water to have a final MeOH concentration of 60%. The final concentration of the I.S. was 60 ng/mL. The remaining 90 μL of the MeOH extracts were stored at −80 °C for subsequent analysis, if necessary.

2.3.3. Column-switching

The analytical process consisted of three steps: loading, transfer and analysis (Table 1). The mobile phases, set at a flow rate of 400 μL/min, were composed of different mixtures of MeOH and water as indicated further, both containing 0.02% formic acid and 1 μmol/L sodium formate. In the first step, following sample injection (40 μL), the proteins and hydrophilic compounds were eluted to waste with 5% MeOH, while the compounds of interest were retained on the extraction column. After 1.0 min, the valve was switched and the analytes trans-

Table 1
Column-switching parameters

Time (min)	Pump 1 (400 μL/min)	Pump 2 (400 μL/min)	Column-switching	Step
0.0–1.0	5% MeOH	65% MeOH	Configuration A	(1) Loading
1.0–1.7	5% MeOH	65% MeOH	Configuration B	(2) Transfer
1.7–6.7	100% MeOH	Gradient 65–90%	Configuration A	(3) Analysis, washing
6.7–16.7	5% MeOH	MeOH		and re-equilibration
16.7–20.7		90% MeOH		
20.7–26.0		100% MeOH		
26.0–31.0		65% MeOH		

ferred in the back-flush mode onto the analytical column with 65% MeOH. At time 1.7 min, the valve was switched to its initial position and analytes were separated using a gradient of MeOH. The analytical and extraction columns were washed with pure MeOH and re-equilibrated with the initial mobile phases.

2.4. MS analysis

Nitrogen was used as nebulizing gas at a pressure of 40 psi (1 psi = 6894.76 Pa) and as drying gas at 9 L/min and at 300 °C. The capillary voltage was set at 3500 V and the fragmentor voltage at 250 and 200 V for CsA and 27-demethoxy-sirolimus, respectively. The MS was set in selected ion monitoring (SIM) mode to detect the sodiated ions $[M + Na]^+$ of CsA (*m/z* 1224.7) and 27-demethoxy-sirolimus (*m/z* 906.5) with a dwell time of 430 ms on each mass.

2.5. Calibration and quality control (QC) samples

2.5.1. Preparation of blank PBMCs

Blank PBMCs were isolated from buffy coats (60 mL) mixed with 70 mL of PBS. In four tubes containing 17 mL of Ficoll-Paque™ Plus solution each, a 32 mL aliquot of cells suspension was added carefully without mixing. After centrifugation at 800 × *g* (20 min at RT), the PBMC layers (found at the interface between the plasma and the Ficoll-Paque™ Plus solution) were collected and pooled in two tubes (2 layers/tube). The cells were washed with 45 mL of PBS (centrifugation at 800 × g for 10 min at RT). The two pellets were re-suspended each in 45 mL of PBS and pooled. Two aliquots of cells suspension (300 μL) were taken out for cell counting. The PBMC suspension was then aliquoted (about 80 aliquots of 7×10^6 cells). After centrifugation at 400 × *g* for 10 min at RT, the supernatants were discarded and the pellets stored at −80 °C.

2.5.2. Preparation of calibration and QC samples

Stock solutions of CsA and 27-demethoxy-sirolimus (I.S.) were prepared in MeOH at a concentration of 100 μg/mL and stored at −80 °C. Further dilutions were achieved in MeOH and blank PBMC samples spiked fresh with 1400 μL of CsA solution at different concentrations. These samples were then processed similarly to the real samples and final concentrations of 5, 75, 200 and 400 ng/mL were reached for the calibration samples and 5, 20, 100, 250 and 400 ng/mL for the QC samples, each containing 60 ng/mL of I.S. and 60% MeOH. A QC sample at 800 ng/mL was prepared and diluted to validate the possibility of sample dilutions, if concentrations fell out of the calibrated range.

2.6. Method validation

The validation strategy was based on the recommendations of the "Société Française des Sciences et Techniques Pharmaceutiques" (SFSTP) [34–36]. The calibration ($k = 4$) and the QC ($k = 5$) samples, were prepared in duplicate ($n = 2$) on each validation day. The concentration range (5–400 ng/mL) was selected on the basis of preliminary results to cover CsA expected levels in clinical samples. A total of 8 validation days were performed, to monitor inter-day variability [37]. The suggested 8 days × 2 replicates per concentration levels are in line with the ISO and FDA recommendations. This validation design is more balanced than the more classical one (3 days × 4 replicates) with higher number of observations and homogenous degrees of freedom for precision variance estimation. Here, degrees of freedom are quite similar for both repeatability and intermediate precision (8 and 7).

Method selectivity was assessed by analyzing blank and spiked PBMC extracts from 6 different healthy volunteers. Calibration curves were based on the peak area ratio of CsA versus the I.S. The trueness, repeatability and intermediate precision were determined with recalculation of the QC samples with the daily response function established. Trueness was expressed as the ratio between theoretical and the average measured concentration. Repeatability was expressed as the coefficient of variation (%CV) of the ratio of the intra-day variance on the theoretical value at each concentration level. Intermediate precision was expressed as the %CV of the ratio of the inter-day variance on the theoretical value at each concentration level. Both variances were obtained using an analysis of variance (ANOVA) as described by Hubert et al. [35]. The LOQ was determined to be the lower QC sample with an acceptable trueness, repeatability and intermediate precision. Previous works have shown that CsA had good stability [38–41], therefore, no further stability studies were performed here.

3. Results and discussion

3.1. Isolation, purification and counting of PBMCs

The use of BD Vacutainer® CPT™ tubes provided an easy handling method for PBMC isolation from whole blood. An important issue is that the procedure must be performed at 4 °C in order to block CsA efflux out of the cells. During method development, PBS washing solutions were retained in order to check the presence of CsA. Aliquots of the PBS washing solutions (1 mL) were evaporated to dryness under vacuum, reconstituted with MeOH containing the I.S., diluted and injected onto the LC–MS system. No residual CsA was detected in the second PBS washing solutions, indicating that two washing steps were sufficient for extensive removal of extracellular CsA from the plasma fraction and that no drug leaked out of the cells during the washing procedure.

Cell counting with a Sysmex® XE-2100 instrument was a rapid and simple method. The variability of this instrument was assessed by preparing PBMC suspensions at three different concentrations in PBS and counting eight aliquots for each cell suspension. The variability obtained, expressed as %CV, was found to be dependant on cell concentrations. Results were satisfactory with 2.1, 9.5 and 15.0%CV for suspensions of 4.8, 1.8 and 0.7×10^6 cells/mL, respectively. This corresponded to the observed concentrations range obtained with clinical samples.

3.2. CsA extraction from PBMCs

To assess CsA extraction from PBMCs, aliquots of cells isolated from whole blood of a healthy volunteer receiving CsA were extracted under different conditions. Both volume and composition of the extraction solvent were evaluated by comparing agitation for 2 h at RT with 300 μL of MeOH, 1400 μL of MeOH and 1400 μL of ethanol. To assess the relative recoveries obtained after this single extraction step, two other extractions under more drastic conditions were performed (agitation with 1400 μL of the respective solvents for 1 h at 40 °C in an ultrasonic bath). The extractions were performed in triplicates and prepared similarly for injection onto the LC–MS system. The quantity of CsA extracted was assessed by calculating the peak area ratio of CsA versus the I.S. The relative recovery of each extraction step was determined by calculating the ratio of the quantity extracted in one step versus the total quantity extracted during the entire process (three steps).

After the first extraction step, the best relative recovery was obtained with 1400 μL MeOH (98 ± 0.6%), followed by 1400 μL ethanol (95 ± 1.5%) and 300 μL MeOH (92 ± 0.6%). In all cases, only small amounts of CsA were extracted during the second step, and no drug was present in the last extraction step, indicating that a single extraction step was sufficient. Based on these results, agitation with 1400 μL of MeOH for 2 h at RT was selected.

3.3. On-line solid-phase extraction

Three steps are generally involved in the development of an on-line solid-phase extraction method: evaluation of sample washout on the extraction column, verification of analyte retention on this column and assessment of transfer from extraction to analytical columns. First, ultraviolet detection at 280 nm was used to assess sample washout. Only 1.0 min was required to elute the endogenous material to the waste with a mobile phase composed of 5% MeOH and a flow rate of 400 μL/min. For analyte retention, different injection volumes (5, 20 and 40 μL) and sample solvent composition after reconstitution (40%, 60%, 80% and 100% MeOH) were evaluated. These parameters must be carefully adjusted because a high MeOH content can result in loss of analyte while a too low MeOH concentration could lead to drug solubility problems. A higher MS signal and a good peak shape were observed with 40 μL injection and 60% MeOH. Finally, a proportion of 65% MeOH was found to be the best compromise between a rapid transfer of the analytes (0.7 min) and an appropriate hydrophobic retention in the analytical column, chosen to be more retentive (C18) than the extraction support (C8).

3.4. Chromatographic separation

The retention times were 9.2 min for the I.S. and 12.3 min for CsA (Fig. 3). A small peak at 13.2 min was observed at the same *m/z* value as CsA. A relatively slow mobile phase gradient from 65% to 90% MeOH in 15 min was required to separate this compound from CsA. This peak has been attributed to an endogenous

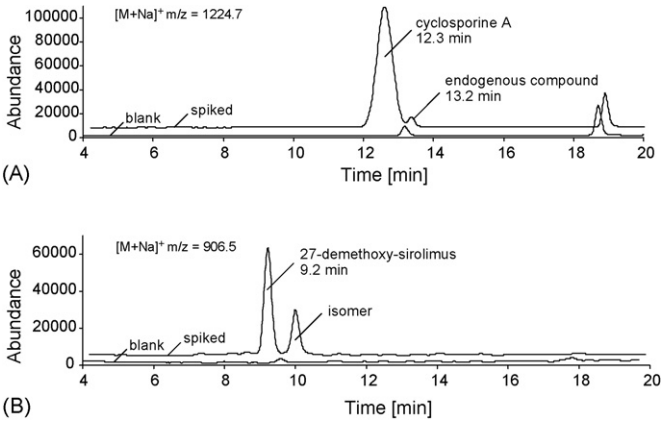

Fig. 3. Chromatograms of a PBMC extract blank and spiked with CsA (50 ng/mL) and I.S. (60 ng/mL). The MS signals at *m/z* 1224.7 (A) and 906.5 (B) are shown.

compound, due to its presence in blank PBMC extracts. To check that this compound was not due to the BD Vacutainer® CPT™ tubes, 60% MeOH was mixed inside the tubes and the solutions analyzed. No peak was observed at 13.2 min. In previous experiments, cyclosporine D (methyl-CsA) was initially evaluated as I.S. This compound was discarded due to a bad observed efficiency on the selected analytical support and therefore relatively high signal to noise ratio. 27-demethoxy-sirolimus was found to be a satisfactory I.S. This compound eluted in a double peak pattern, attributed to *cis*- and *trans*-isomers, as shown in previous studies [42,43]. Only the main peak was used for quantification. The total time for the analysis of one sample was 31.0 min, including washing and re-equilibration of the system.

The use of an automated on-line solid-phase extraction system presents the advantage to be a rapid method comparatively to off-line sample preparation methods and could also compensate for the relatively long PBMC isolation and CsA extraction steps. Compared to a direct injection procedure, the use of a column-switching system yields a cleaner sample for injection onto the analytical column and allows analyte preconcentration. The presence of other compounds eluting simultaneously with CsA at different *m/z* ratio was verified in scan mode (*m/z* 500–1500) with injection of PBMCs extracts spiked with CsA. No other compounds with important signal co-eluted with this drug at 12.3 min. In the present study, the ion suppression was expected to be limited. The sample pre-treatment, protein precipitation technique with MeOH, has been shown to remove up to 92% of the proteins present in human plasma [44]. Therefore, only a small residual protein amount was supposed to be present during on-line extraction. Residual proteins should be discarded to the waste during the on-line extraction process. As apparent retention factors were important for the monitored analytes, the latter were eluted after the matrix effect time window described by Marchi et al. in case of similar configuration [45].

3.5. MS results

Although, the use of LC–MS/MS is increasing in bioanalytical chemistry, a compromise has to be found between method

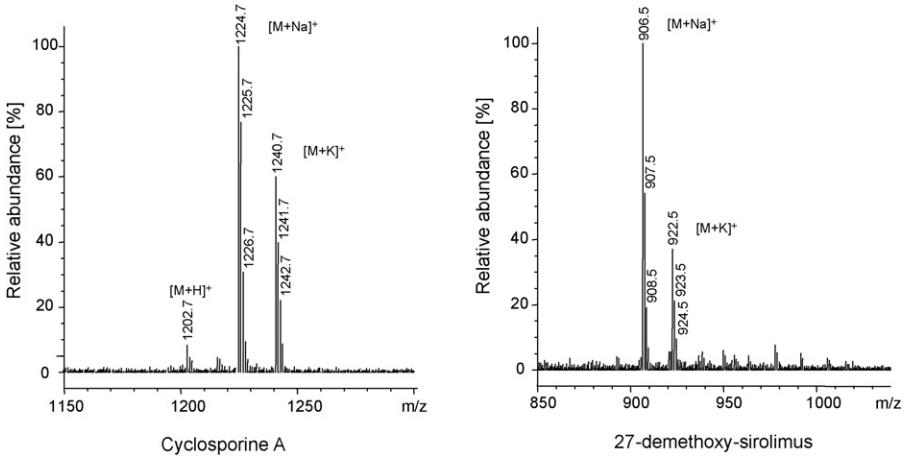

Fig. 4. Mass spectra for the studied compounds. 100 ng/mL solutions (10 μL) in MeOH/water (8/2) containing 0.02% formic acid and 1 μmol/L sodium formate were injected directly into the mass spectrometer.

complexity and ease-of-use. Several methods have recently been published for CsA quantification in whole blood in LC–MS/MS [20–23], however for TDM the use of single quadrupole MS method could be considered. The latter is simple, less expensive and can be used in most clinical laboratories with very good performance. It has to be noted that the main drawback of single quadrupole MS strategies is related to the fact that only one ion in SIM mode is generally used for drug quantification [25,41], which could be insufficient for method selectivity, particularly when low molecular weight compounds are monitored. Here, with compound molecular mass higher than *m/z* 900, this issue appeared less critical. Both CsA and I.S. were mainly detected as their sodium adduct ions $[M + Na]^+$ (Fig. 4). Such adducts were regularly selected by other groups that have quantified these compounds with a similar detector [24,39,41,46,47]. Potassium adduct ions $[M + K]^+$ were also observed for CsA and I.S. and protonated molecule $[M + H]^+$ was observed for CsA. Some authors have proposed to regulate multimer formation with the incorporation of a primary amine (dodecylamine) in the mobile phase [48], but this alternative failed to form one dominating species in independent experiments achieved for CsA. Therefore, the monitoring of the sodium adduct ion at *m/z* 1224.7 and 906.5 were found to be the best compromise and further evaluated for the quantitative studies. Here, non-optimal selectivity issue with only one ion monitored per compound was considered acceptable thanks to an absence or identified co-medication in the tested samples.

3.6. Validation results

The method was selective and no interferences were observed at the retention time of CsA and I.S. with six different blank PBMC extracts. To determine the best response function, different regression models (linear regression, linear regression through 0, linear regression on square-root transformed data, linear regression on log transformed data, quadratic regression and weighted linear regression (weighted factor $1/x$ or $1/x^2$)) were evaluated based on the total error concept proposed by SFSTP and the tolerance intervals evaluation. The best calibration model, giving the highest quality results using accuracy profiles (Fig. 5), was the log-transformation of the variables. The accuracy profile was built using trueness and intermediate fidelity variance as indicated by Boulanger et al. [36]. A 30% relative error was selected as the acceptance threshold where the expected proportion of measures (95%) that will fall within the acceptance limits. It must be noted, that FDA recommenda-

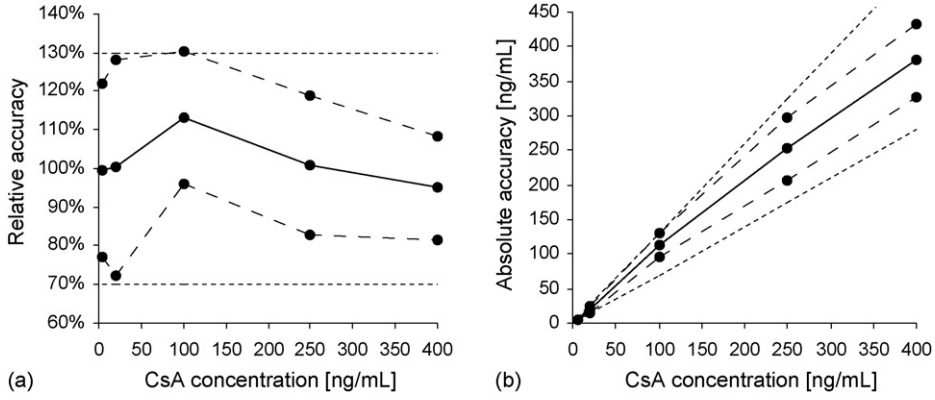

Fig. 5. Relative (A) and absolute (B) accuracy profiles for CsA with log transformed data. The solid line indicate the trueness and the dashed lines represent the accuracy calculated as trueness plus and minus 1.89 times intermediate precision. The dotted lines represent the acceptance limits of ±30%.

Table 2
Trueness, repeatability and intermediate precision for CsA

CsA concentration (ng/mL)	Trueness (%)	Precision	
		Repeatability (%)	Intermediate precision (%)
5	99.7	9.9	11.8
20	100.3	9.6	14.7
100	113.2	5.1	9.1
250	100.9	7.2	9.5
400	95.0	5.2	7.0
800/2	91.8	5.0	8.5

tions for bioanalysis (15% bias and 15% precision) lead to an acceptance level of about ±40%. The LOQ was 5 ng/mL and corresponds to the lower QC with acceptable validation results. On the evaluated assay range, trueness, repeatability and intermediate precision were found to be satisfactory (Table 2). For samples with concentrations higher than 400 ng/mL, the performance of the diluted 800 ng/mL QC was not statistically different from the undiluted 400 ng/mL concentration level (Student *t*-test ($\alpha = 0.05$), data not shown). Therefore, when out-of-range concentrations are observed within the routine use of the method, a simple dilution of the sample can be performed.

3.7. Application of the LC–MS method

This method was successfully applied in two pharmacogenetic clinical research protocols approved by local Ethics Committees involving healthy volunteers (Ansermot et al., submitted) and transplant patients (Crettol et al., submitted). The pharmacokinetics of CsA within PBMCs and the influence of *ABCB1* gene polymorphisms on the intracellular CsA distribution were evaluated. Fig. 6 shows an example of a chromatogram of PBMC extracts from a healthy volunteer 2 h after intake of a single oral dose of CsA (2 mg/kg). The injected drug concentration measured by LC–MS was 208 ng/mL and the number of PBMCs in the sample was 5.2×10^6, which corresponds to a CsA amount of 9.3 fg/PBMC.

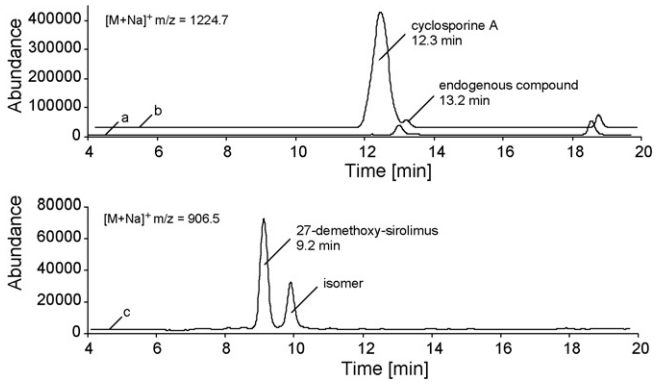

Fig. 6. Chromatograms of PBMC extracts of a healthy volunteer before (a) and 2 h after (b) intake of a single oral dose of CsA (2 mg/kg). Chromatogram of the I.S. (c). In this example, the injected concentration measured was 208 ng/mL and the number of PBMCs in the sample was 5.2×10^6, which corresponds to a CsA amount of 9.3 fg/PBMC.

4. Conclusions

A sensitive and selective analytical method was developed for the quantification of CsA in human PBMCs with on-line solid-phase extraction coupled with LC-ESI-MS. To our knowledge, this is the first method using this technique for the intracellular quantification of CsA. This method is very sensitive and may present better selectivity than immunoassays. The developed method showed good performance in terms of trueness, repeatability and intermediate precision. The CsA intracellular concentrations measured included the amount present in the cytoplasm and in the membranes of the PBMCs. The measured levels are expected to be more representative of CsA concentration at the site of action than the measurement in whole blood. Despite the time needed for PBMC isolation and CsA extraction, this method is a new potential tool for TDM of CsA and could be used in clinical conditions if the utility of intracellular measurements is confirmed. Prospective studies are needed to evaluate the correlation between intracellular concentrations and clinical impact, and intracellular therapeutic indexes should be established.

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