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Determination of ciclosporin A and its six main metabolites in isolated T-lymphocytes and whole blood using liquid chromatography–tandem mass spectrometry

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Abstract

A specific and sensitive method for determination of intracellular ciclosporin A (CsA) and its six main metabolites AM1, AM9, AM1c, AM1c9, AM19 and AM4N, in isolated T-lymphocytes and whole blood is described. T-lymphocytes were separated from whole blood using Prepacyte®. The analytes were extracted and purified from isolated lymphocytes and whole blood by protein precipitation followed by solid-phase extraction (SPE). The analytes and the internal standard, ciclosporin C (CsC), were separated on a reversed phase C8 column (30 mm × 2.1 mm, 3 μm) with a 10 mm × 2 mm, 5 μm Drop-In Guard Cartridge, using gradient elution chromatography and tandem ion trap mass spectrometry detection. The method has been validated in accordance with FDA guidelines and showed linear range from 0.25 to 500 ng/mL for CsA, 0.5 to 500 ng/mL for AM1, AM9 and AM19, 1 to 500 ng/mL for AM4N, AM1c and AM1c9 in intracellular matrix, and 2.5 to 3000 ng/mL for all analytes in whole blood. The applicability of the method is shown on patient samples.

Keywords: Cyclosporine; Lymphocytes; Mass spectrometry; Intracellular; Metabolites; T-cells

1. Introduction

Ciclosporin A (CsA) is a cornerstone of the immunosuppressive therapy in solid organ transplant recipients and has resulted in increased survival rates of both patients and grafts since its introduction in the market in the early 80s [1,2]. In 2003, 77% of all kidney transplant recipients in Norway were treated with CsA [3]. However, CsA treatment is associated with serious side-effects such as increased blood pressure, development of diabetes, dyslipidemia, intermittent renal hypoperfusion and chronic nephrotoxicity [4–6]. The pharmacokinetics of CsA exhibits extensive inter- and intrapatient variation, and CsA has a narrow therapeutic window with serious consequences when over- and underdosed. Due to this, routine therapeutic drug monitoring of CsA is necessary.

All routine monitoring is optimally performed in whole blood due to the distribution of CsA into erythrocytes, which is temperature and concentration dependent [7,8]. The immunosuppressive site of action for CsA is inhibition of the intracellular phosphatase calcineurin in T-lymphocytes [9].

CsA is both a substrate and an inhibitor of the transmembrane transporter P-glycoprotein (P-gp), which is expressed in the T-lymphocytes and transports CsA out of the cells [10,11]. The expression of P-gp will therefore influence the intracellular concentration of CsA and affect its pharmacodynamic effect. An up-regulation of P-gp following transplantation has been shown in lymphocytes from renal transplanted patients [12]. Measurement of intracellular CsA concentration in T-lymphocytes could therefore give more relevant information with regards to efficacy of CsA as compared to whole blood concentrations. Previous work has shown interesting results on the correlation between

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clinical effect and CsA lymphocyte binding [13,14]. However, the lymphocyte separation technique referred to in those studies (*Fiqoll centrifugation*) is relatively unspecific and does not allow selective isolation of T-lymphocytes [15].

The cytochrome P-450 3A (CYP 3A) subfamily is responsible for metabolizing CsA to more than 30 metabolites [16]. Generally, the metabolites are considered to have both less immunosuppressive effect and less toxic effect than CsA [17,18]. However, some data indicate that the secondary metabolites AM19 and AM1c9 are more nephrotoxic than CsA and other metabolites [19,20]. Studies also indicate higher formation of the secondary metabolites AM19 and AM1c9 in patients with functional CYP3A5 enzymes [21].

The method described in this article is a validated, sensitive analytical tool capable of measuring CsA and six of its main metabolites AM19, AM1c9, AM1, AM9, AM1c and AM4N, intracellularly in T-lymphocytes as well as in whole blood from solid organ transplant recipients. This is the first report of a sensitive analysis of CsA and its main metabolites suitable for routine intralymphocyte measurements. The method will be used to monitor solid organ transplant recipients prospectively after transplantation to elucidate the understanding of the mechanism behind both the lack of immunosuppressive effect during acute rejection episodes and the cause of the nephrotoxic adverse event of CsA.

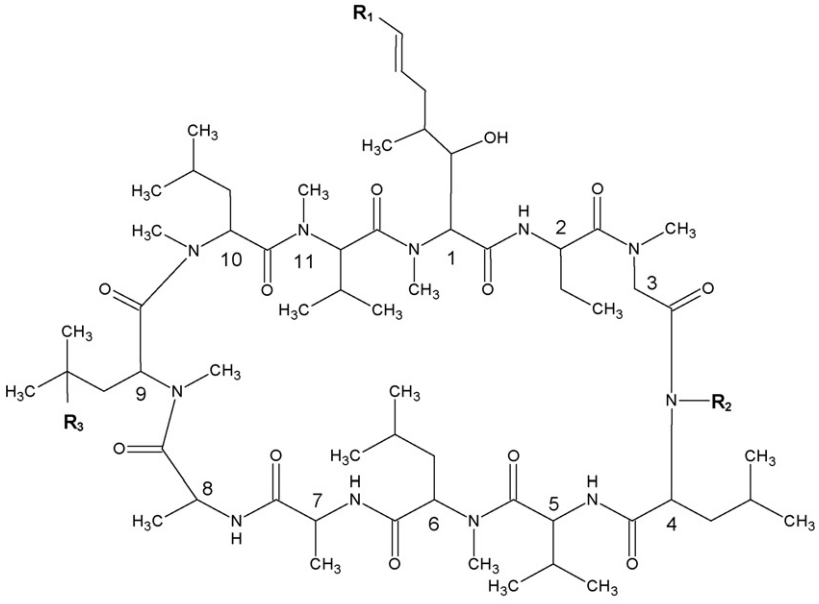
2. Experimental

2.1. Chemicals and reagents

CsA, Ciclosporin C (CsC) and the metabolites AM1, AM9 and AM1c were provided by Novartis (Basel, Switzerland). The metabolites AM4N, AM19 and AM1c9 were kindly provided by Dr. U. Christians (University of Colorado Health Sciences Centre, Denver, USA). Verapamil hydrochloride was purchased from Sigma Chemical, St. Louis, USA. The standard solutions of the metabolites were made by dissolving 5 mg of pure metabolites in 5 mL of methanol. The lymphocyte isolation medium Prepacyte® and erythrocyte lysis medium, VitaLyse™, were purchased from BioErgonomics, St. Paul, USA. All other chemicals used were of analytical grade. The structural formulae of CsA and the six metabolites are shown in Fig. 1.

2.2. Apparatus

The chromatographic system used consisted of a Spectra System P4000 pump and an AS3000 auto sampler (ThermoFinnigan, Austin, USA). The samples were separated on a reversed phase C8 column (30 mm × 2.1 mm, 3 μm) with a 10 mm × 2 mm, 5 μm Drop-In Guard Cartridge (Thermo Electron Corp., CT, USA). The columns were heated to 65 °C in a



	R_1	R_2	R_3
CsA	CH_3	CH_3	H
AM1	CH_2OH	CH_3	H
AM1c	CH_2OH (MeBmt: cyclic)	CH_3	H
AM4N	CH_3	H	H
AM9	CH_3	CH_3	OH
AM19	CH_2OH	CH_3	OH
AM1c9	CH_2OH (MeBmt: cyclic)	CH_3	OH

Fig. 1. Structural formula of CsA ($C_{62}H_{111}N_{11}O_{12}$) and main metabolites.

Perkin-Elmer, Series 200 column oven. The analytes were eluted using a stepwise gradient at the flow rate of 0.2 mL/min with mobile phase A consisting of acetonitrile (ACN)/20 mM ammonium formate buffer ($NH_4^+COO^-$) pH 3.6 (20:80, v/v) and mobile phase B consisting of ACN/20 mM $NH_4^+COO^-$ (80:20, v/v). The water phase of the mobile phase was made by adding formic acid to 20 mM of ammonium solution in order to achieve pH 3.6. The gradient program was as follows: 65% mobile phase A for 20 min followed by a linear increase of mobile phase B to 80% for 10 min, and finally 8 min of re-equilibration at start conditions.

2.3. MS–MS configuration

The liquid chromatography (LC) apparatus was connected to a Finnigan LCQ^{DUO} ion trap tandem mass spectrometry (MS–MS) detector (ThermoFinnigan) using an atmospheric pressure chemical ionization (APCI) interface. The detector was operated in positive ion mode. Data calculation and system control was done with Xcalibur Version 1.3 (ThermoFinnigan). Tuning of the (MS–MS) detector and optimization of the APCI operating conditions were performed by direct injection of each analyte (5 μg/L in 65% mobile phase A and 35% mobile phase B). The optimal APCI conditions determined was a vaporizer temperature of 400 °C, a heated transfer capillary temperature of 220 °C and voltage of 46 V, a discharge current of 6 μA and a sheath gas flow of 60 units and aux gas flow at 5 units (arbitrary software unit). The tube lens offset was 10 V, and the scan cycle was 200 ms. All analytes were separated chromatographically to minimize cross-talk between the highly resembling analytes. For MS–MS each transition was optimized on collision energy, and each segment was optimized to maximize the signal intensity. All daughter ions that showed more than 30% signal of the main fragment intensity were included in the MS–MS quantification, see Table 1.

2.4. Lymphocyte isolation

T-lymphocytes were isolated with Prepacyte® from freshly drawn heparine whole blood according to the standard procedures from the manufacturer. An aliquot of 100 μM of verapamil was added to the heparine tube before blood sampling to inhibit P-gp from pumping CsA out of cells before analysis [22]. Prepacyte® uses negative separation: antibodies against specific antigens expressed on the surfaces of erythrocytes, B-lymphocytes, mature myeloid cells like granulocytes and monocytes and platelets, causing agglutinating of these cells, but not the T-lymphocytes. To remove excess erythrocytes from the supernatant, Vitalyse^{TM} was added to lyse these cells. After two rounds of centrifugation (400 *g*) and washing, the remaining supernatant contains >97% lymphocytes with T-lymphocytes comprising 88–96% of the resultant cell population [23]. The isolating method starts with 7 mL of whole blood and produces a T-lymphocyte isolate pellet that is finally added 1 mL methanol:ACN:water (1:1:3) for cell lysis and protein precipitation. The mixture was stored at −30 °C until solid-phase extraction (SPE) and subsequent analysis.

During optimization of the T-lymphocyte separation method, it was determined that the lymphocytes need to be isolated from whole blood within 4 h to avoid leakage of CsA out of the cells. Adding verapamil to avoid P-gp pumping of CsA out of the cells during sample handling gave significantly higher intracellular concentrations compared to no P-gp inhibition (data not shown).

2.5. Whole blood treatment

Aliquots of 1 mL whole blood containing heparine and verapamil were stored at −30 °C until analysis. To a volume of 0.20 mL whole blood was 50 μL of 1.0 μg/mL CsC (in methanol:ACN:water (1:1:3)) added before mixed with 0.60 mL methanol for protein precipitation. The supernatant

Table 1
An overview of the segments, fragmentation transitions and optimal collision energy of the eight analytes in the MS–MS configuration

Segment	Time (min)	Analyte	Parent ion $[M+H]^+$	Collision energy (%)[a]	Daughter ion $[M+H]^+$
1	0–13	AM19	1235.8 ± 4	28	995.4 ± 3 1090.5 ± 3 1132.6 ± 3 1217.5 ± 3
		AM1c9	1235.8 ± 4	28	1059.5 ± 3 1090.6 ± 3 1191.7 ± 3
2	13–25	AM1 and AM9	1219.7 ± 4	30	1074.6 ± 3 1077.7 ± 3 1116.7 ± 3 1201.7 ± 3
		AM1c	1219.7 ± 4	32	1106.7 ± 3 1188.7 ± 3
3	25–35	AM4N	1189.7 ± 4	28	1171.7 ± 3
		CsC	1219.7 ± 4	32	1201.7 ± 3
		CsA	1203.7 ± 4	28	1101.7 ± 3 1185.7 ± 3

[a] The relative collision energy corresponds to 0–5 V peak-to-peak of resonance excitation RF voltage.

was centrifuged for 15 min at 12,500 × *g*, and to minimize pollution from the pellet the supernatant was transferred into a new vial with a second centrifugation step (15 min at 12,500 *g*). The final supernatant was added 0.30 mL water before the standard SPE procedure was completed. Total organic modifier concentration was below 55% before SPE extraction.

2.6. Standard SPE procedure

The isolated cells and whole blood was thawed at room temperature. An aliquot of 50 μL of 1.0 μg/mL CsC (in methanol:ACN:water (1:1:3)) was added to the matrix. After protein precipitation and centrifugation (12,500 × *g* at 7 min) the supernatant was applied on the pre-conditioned SPE column (Water Oasis®, HLB 1 cc, 30 mg) (preconditioning: 1 mL methanol followed by 1 mL water). The column was then washed with 1 mL water and 1 mL 65% methanol before eluting with 1 mL 90% methanol. The elute was evaporated until dryness using a Speedvac® (Thermo SPD12IP), and reconstituted in 0.25 mL of 65% mobile phase A and 35% mobile phase B, followed by a last centrifuge step before injecting 100 μL of the supernatant on the LC–MS/MS system.

2.7. Determination of the optimal SPE eluting conditions

To determine the optimal wash and eluting conditions, a sample containing 500 ng of each of the analytes was applied on the SPE column before eluting with increasing methanol strength (20–100%) followed by measuring the content of the elute. In a second experiment, the optimal combination of methanol and ACN to be used for protein precipitation was determined. This combination should at the same time give maximum retention on the SPE column. The condition that gives these two properties was determined as follows: 500 ng of each of the analytes was applied on the SPE column followed by eluting with 20% methanol/*x*% ACN, where *x* ranges from 0 to 80%. The highest ACN concentration at which all analytes still were retained was used to precipitate the proteins in the intracellular matrix. After centrifugation (12,500 × *g* at 5 min) the supernatant was applied directly on the preconditioned SPE column.

2.8. Cell count and protein determination

To relate the intracellular concentrations to a relevant physiological parameter, both cell counts of isolated T-lymphocytes using a *Bürker chamber* and protein measurements were performed. The protein concentration was determined by adding 160 μL of 25% Coomassie Brilliant Blue G-260 to 50 μL of the cell suspension, followed by monitoring of the absorption switch from 465 to 595 nm using Wallac 1420 Victor3^{TM} (Perkin-Elmer Corp.). The calibration curve for protein determination was produced with bovine protein on each analysis day.

2.9. Validation

The validation of the assay was based on the Guidelines for Bioanalytical Method Validation from FDA [24].

Lower limit of quantification (LLoQ) was determined using the following criteria: an analytical response of at least five times the blank response, an identifiable analytical peak, discrete and reproducible peak with precision CV <20% and an accuracy of 80–120%. Precision for intra-day variation and accuracy was determined with five replicates at low, medium and high concentration. Inter-day precision was calculated using three replicates at low, medium and high concentration for 5 following days.

Samples for the calibration curves were produced by adding known concentrations of CsA and the six metabolites with CsC as internal standard to the respective drug free blank matrix. All calibration curves comprised of at least 10 concentrations, including a blank sample. Each concentration point of the curve was done in duplicates, except for one low, one middle and one high concentration from the intra-day precision (see Table 3 for details), which was done in five replicates and also included in the calibration curve. In addition a zero-sample (with internal standard) was tested.

The recovery of the SPE procedure was determined by adding standards in different concentrations (low, medium and high, see Table 3) in two replicates to blank matrix solution before and after the SPE procedure, respectively.

Specificity was determined by analysing for interfering substances in blank samples without CsA or metabolites of both whole blood and cell isolates from six healthy volunteers and two kidney transplant recipients who were not receiving CsA treatment. The healthy volunteers were not taking any drugs, and the kidney transplant recipients were not using CsA but all other commonly used drugs in this patient population.

The matrix effect on the determination of the analytes was evaluated by comparing three different standard concentrations (low, medium and high) in mobile phase with the same concentrations added to blank matrices after these were subjected to standard SPE sample preparation.

The stability of both stock solutions and biological standard samples of CsA and all metabolites was tested. For this purpose two replicates of low (1 ng/mL for intracellular, 5 ng/mL for whole blood) and high (100, 500 ng/mL, respectively) concentrations were tested for short-term stability by storing standard solutions for 24 h at −30 °C followed by 24 h at room temperature ($n = 4$), post-preparative in auto sampler for 24 h ($n = 4$), three freeze/thaw cycles after 1, 2 and 3 days at −30 °C ($n = 6$). Long-term stability was tested by storing both low and high concentrations at −30 °C for 2, 5, 8 and 14 weeks ($n = 8$). Stock solutions were tested for stability in ambient laboratory temperature for 12 h.

3. Results and discussion

3.1. MS–MS conditions

To optimize detection, the chromatogram was divided in three segments and each segment consisted of two or more scan events (Fig. 2C and Table 1). All analytes were detected with unique scan event, except AM1 and AM9 as these two analytes produced identical daughter ions after fragmentation. Optimal sensitivity for the analytes was obtained by multiple reaction monitoring.

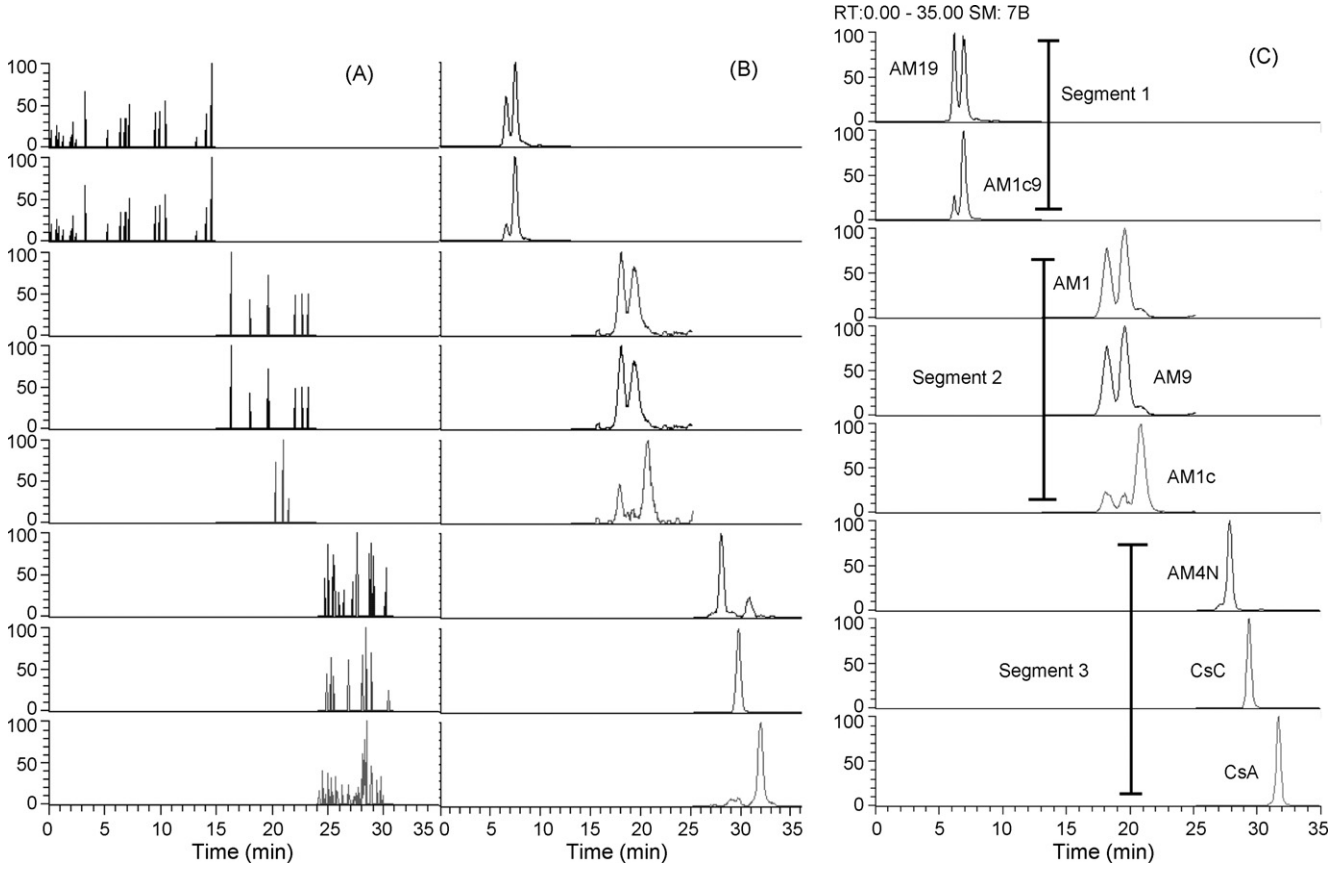


Fig. 2. Three different MS–MS chromatographies from blank patient sample (A), standard sample at LLoQ (B) and representative patient sample from a pharmacokinetic study (C). The chromatogram shows all mass transition divided in the segments and scan events as described in Section 3.1, and Table 1. Elution order with corresponding whole blood concentration in ng/mL in prentices: AM19 (21.9), AM1c9 (29.7), AM1 (787.5), AM9 (505.6), AM1c (105.5), AM4N (169.9), CsC, CsA (1405.8). The signal strength in (A) is 1/100 of the signal strength at LLoQ (B).

The sum of signals of the daughter ions listed in Table 1 was used for quantification.

3.2. Optimal SPE conditions

Fig. 3A shows a plot of analyte recovery against the amount of methanol used in the washing step. The maximum methanol strength of the washing solution that did not result in elution of analytes from the SPE column was determined to be 65%. To obtain high and reproducible recovery, 90% methanol was needed in the elution step. The optimal combination of ACN and methanol that still gave full retention on the SPE column was determined to be 20% ACN and 20% methanol in water (Fig. 3B). This solution mixture was therefore used to precipitate the isolated lymphocytes so that the stored supernatant of the isolate could be applied directly on the SPE column.

3.3. Validation

The calibration curve covered a wide range of concentrations, thereby making it necessary to divide the curve in one low and one high concentration interval. Table 2 shows the equations and

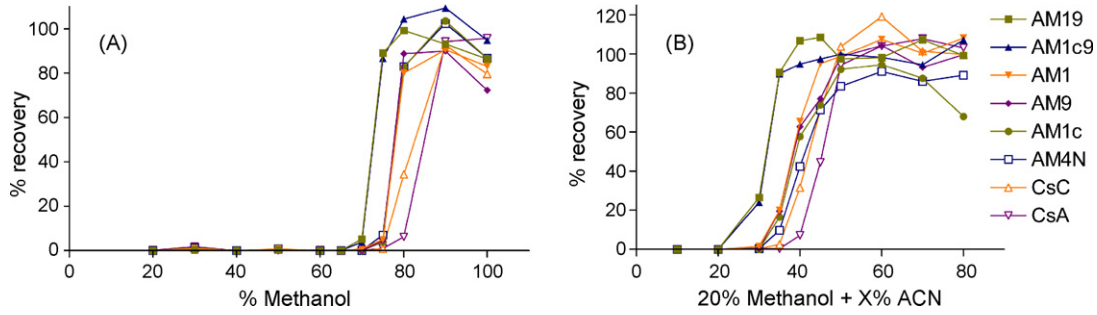


Fig. 3. Left figure (A) shows the eluting of all eight analytes when increasing the methanol concentration. Methanol could be increased to 65% before any breakthrough was observed. The right figure (B) shows the determination of the optimal precipitation condition. A fixed amount of 20% methanol was added ACN in increasing volume percentage. The optimal condition for maximum precipitation and full retention on the SPE column was methanol:ACN:water (1:1:3).

Table 2
All standard curve equations ($y = ax + b$) for low and high concentration for both intralymphocyte and whole blood

	Intralymphocyte	Whole blood
CsA	0.25–10 ng/mL: $y = 0.00897x - 0.0065$ ($r^2 > 0.999$) 10–250 ng/mL: $y = 0.00802x + 0.2041$ ($r^2 > 0.999$)	2.5–50 ng/mL: $y = 0.0176x - 0.0051$ ($r^2 > 0.996$) 50–3000 ng/mL: $y = 0.0143x + 0.219$ ($r^2 > 0.996$)
AM9	0.5–10 ng/mL: $y = 0.0093x - 0.0005$ ($r^2 > 0.999$) 10–250 ng/mL: $y = 0.01363x - 0.1395$ ($r^2 > 0.997$)	2.5–50 ng/mL: $y = 0.0055x + 0.0009$ ($r^2 > 0.994$) 50–3000 ng/mL: $y = 0.0045x + 0.0685$ ($r^2 > 0.991$)
AM19	0.25–10 ng/mL: $y = 0.0499x - 0.0020$ ($r^2 > 0.999$) 10–100 ng/mL: $y = 0.0595x - 0.2753$ ($r^2 > 0.995$)	2.5–50 ng/mL: $y = 0.0066x - 0.0035$ ($r^2 > 0.998$) 50–500 ng/mL: $y = 0.0052x - 0.063$ ($r^2 > 0.990$)
AM1c9	0.25–10 ng/mL: $y = 0.00723x - 0.0052$ ($r^2 > 0.999$) 10–100 ng/mL: $y = 0.00758x - 0.1439$ ($r^2 > 0.999$)	2.5–50 ng/mL: $y = 0.0104x - 0.0050$ ($r^2 > 0.987$) 50–500 ng/mL: $y = 0.0097x + 0.0317$ ($r^2 > 0.989$)
AM1	0.5–10 ng/mL: $y = 0.0053x - 0.0003$ ($r^2 > 0.999$) 10–250 ng/mL: $y = 0.0098x - 0.1432$ ($r^2 > 0.992$)	2.5–50 ng/mL: $y = 0.0031x - 0.0004$ ($r^2 > 0.998$) 50–3000 ng/mL: $y = 0.0025x + 0.0458$ ($r^2 > 0.999$)
AM1c	0.5–10 ng/mL: $y = 0.0045x + 0.0009$ ($r^2 > 0.997$) 10–250 ng/mL: $y = 0.0064x - 0.0528$ ($r^2 > 0.997$)	2.5–50 ng/mL: $y = 0.0015x + 0.0007$ ($r^2 > 0.987$) 50–3000 ng/mL: $y = 0.0014x + 0.0628$ ($r^2 > 0.998$)
AM4N	1–10 ng/mL: $y = 0.00436x + 0.0007$ ($r^2 > 0.998$) 10–100 ng/mL: $y = 0.0460x - 0.0522$ ($r^2 > 0.997$)	2.5–50 ng/mL: $y = 0.0101x + 0.0015$ ($r^2 > 0.996$) 50–500 ng/mL: $y = 0.0106x + 0.0035$ ($r^2 > 0.999$)

Each analyte in one concentration range has one calibration curve. r^2 are shown in parentheses. The unit for intralymphocyte is ng of analyte per mL of isolated lymphocytes from healthy volunteers. During cell separation 7 mL of whole blood produces 1 mL of isolated lymphocyte suspension. The lowest concentration for each analyte represents the LLoQ for corresponding matrix.

their regression coefficients (r^2) which were >0.99 in the complete interval. The lowest concentrations in the calibration curve for each analyte are the respective LLoQ values. Table 3 shows the data for recovery, inter- and intra-day precision and accuracy for all analytes in both intracellular and whole blood. All data were within the limit of acceptance of the FDA guidelines. The accuracy of CsA was validated against concentration measurements done at the accredited laboratory of the Rikshospitalet, University of Oslo in addition to in-house controls. The accuracy for the CsC, AM1 and AM9 was determined after several weighting of pure metabolite powder provided by Novartis. For the metabolite AM19, AM1c9 and AM4N the accuracy was determined after several in-weighing of pure, not certified, metabolite solutions. The CsA and metabolites were all conditions tested as described in Section 2.9. The different concentrations showed no decreasing trend over time, but varied between 81 and 121% of the reference concentration.

Both blood and intracellular matrix from six healthy volunteers (no medications) and two kidney transplant recipients were used to test for interfering substances. Both transplanted patients were treated with prednisolone, mycophenolate mofetil, furosemide and acetylsalicylic acid. In addition, the patients were individually treated with allopurinol, nifidipine, ramipril, ranitidine, atorvastatin, fluvastatin, metoprolol and alendronate. All blood samples were collected on heparine tubes containing 100 μmol verapamil. No interfering peaks were detected.

No interfering matrix effect on the analytes was observed under the conditions described in Section 2.9. The signals for all analytes in mobile phase were relative to signals from standard added to blank matrices after standard SPE within 95.0–114.6%.

3.4. Patient samples

All intracellular concentrations were calculated relative to mg protein, per 10^6 lymphocytes as well as per mL whole blood.

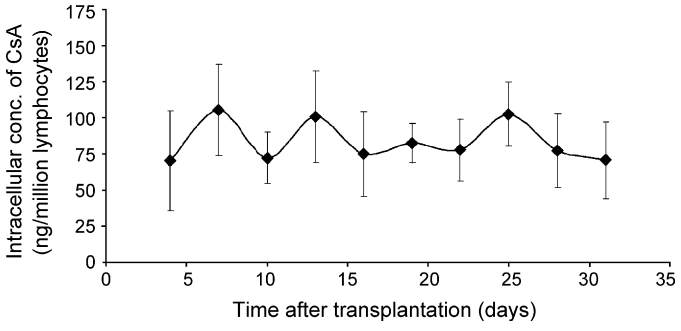


Fig. 4. Mean ± S.E.M. CsA intracellular concentration of CsA per million T-lymphocytes ± S.E.M. in six stable renal transplant patients during the first month after transplantation.

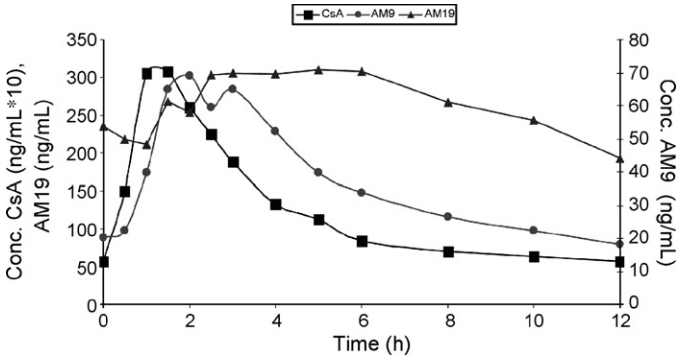


Fig. 5. Average whole blood pharmacokinetic profile of CsA and metabolite AM9 and AM19 from six renal transplanted patients. The concentration of CsA and AM9 is displayed on left axis, and AM19 concentration on right axis. All CsA concentrations are divided by 10. AM9 is a primary metabolite of CsA, while AM19 is a secondary metabolite. The formation of the primary metabolite AM9 has a lag time of approximately 0.5 h compared to CsA, while the secondary metabolite AM19 has a lag time of about 1 h. The concentration of the secondary metabolite AM19 is more constant because more than one primary metabolite could be metabolized to AM19.

Table 3
Intracellular validation is shown in italic, and whole blood validation in normal text

Concentration	Intracellular	Whole blood	Intra-day		Inter-day		Recovery (%)[a]	
			Accuracy (%) ± CV (%)		Accuracy (%) ± CV (%)		Intracellular	Whole blood
			Intracellular	Whole blood	Intracellular	Whole blood		
CsA								
Low	0.25	2.5	*107.1 ± 4.8*	87.6 ± 4.4	*90.2 ± 8.4*	115.8 ± 13.1	*85.5 (0.5)*	119.5 (5)
Middle	10	25	*104.0 ± 3.6*	99.1 ± 10.5	*99.3 ± 2.1*	108.5 ± 14.0	*83.1 (25)*	114.0 (50)
Middle2	–	250	–	109.7 ± 13.9	–	105.8 ± 11.2	–	–
High	100	1500	*99.6 ± 8.9*	102.1 ± 6.5	*100.2 ± 5.3*	98.1 ± 13.0	*93.3 (100)*	112.7 (500)
AM19								
Low	0.25	2.5	*106.8 ± 14.4*[b]	104.2 ± 12.4[b]	*81.9 ± 14.6*	105.6 ± 18.9[b]	*73.5 (0.5)*	114.2 (5)
Middle	10	25	*100.2 ± 14.2*[b]	98.6 ± 14.0[b]	*85.9 ± 4.8*	86.3 ± 14.6[b]	*84.3 (25)*	111.6 (50)
High	100	250	*101.5 ± 12.8*[b]	104.2 ± 11.1[b]	*99.7 ± 5.4*	97.6 ± 14.9[b]	*76.0 (100)*	107.9 (500)
AM1c9								
Low	0.25	2.5	*113.3 ± 10.4*[b]	104.1 ± 12.2[b]	*98.2 ± 14.6*	112.7 ± 19.3[b]	*79.4 (0.5)*	116.2 (5)
Middle	10	25	*100.3 ± 11.7*[b]	100.5 ± 14.5[b]	*85.8 ± 6.1*	110.9 ± 10.8[b]	*82.6 (25)*	113.0 (50)
High	100	250	*99.9 ± 9.8*[b]	98.9 ± 13.8[b]	*99.4 ± 7.8*	101.7 ± 12.4[b]	*80.3 (100)*	106.4 (500)
AM1								
Low	0.5	2.5	*119.5 ± 14.6*	90.7 ± 14.5	*86.0 ± 10.0*	105.5 ± 7.7	*97.9 (0.5)*	112.9 (5)
Middle	10	25	*100.7 ± 11.0*	99.8 ± 13.7	*94.0 ± 7.6*	90.8 ± 14.8	*95.6 (25)*	117.2 (50)
Middle2	–	250	–	100.5 ± 8.9	–	115.8 ± 14.0	–	–
High	100	1500	*102.0 ± 8.4*	100.6 ± 14.3	*97.5 ± 14.7*	99.4 ± 3.7	*92.8 (100)*	108.1 (500)
AM9								
Low	0.5	2.5	*108.9 ± 13.8*	83.2 ± 11.8	*90.1 ± 11.2*	83.0 ± 19.0	*82.0 (0.5)*	103.7 (5)
Middle	10	25	*100.2 ± 12.4*	99.6 ± 13.2	*87.0 ± 3.5*	105.2 ± 13.1	*98.6 (25)*	98.7 (50)
Middle2	–	250	–	104.3 ± 14.8	–	96.2 ± 12.7	–	–
High	100	1500	*101.2 ± 10.3*	97.2 ± 4.7	*98.1 ± 9.5*	102.5 ± 14.1	*92.4 (100)*	109.3 (500)
AM1c								
Low	0.5	2.5	*103.9 ± 13.3*	102.6 ± 11.8	*113. 0 ± 13.7*	107.7 ± 19.7	*94.7 (0.5)*	107.5 (5)
Middle	10	25	*111.7 ± 3.3*	100.3 ± 11.3	*99.1 ± 4.7*	105.3 ± 13.9	*97.0 (25)*	103.6 (50)
Middle2	–	250	–	83.4 ± 13.2	–	113.4 ± 12.8	–	–
High	100	1500	*101.1 ± 10.8*	99.8 ± 14.8	*98.7 ± 7.6*	99.5 ± 8.4	*95.2 (100)*	100.8 (500)
AM4N								
Low	1	2.5	*91.4 ± 9.8*[b]	87.3 ± 13.8[b]	*104.0 ± 12.4*[b]	116.0 ± 3.8[b]	*81.6 (1)*	118.7 (5)
Middle	10	25	*110.4 ± 9.4*[b]	99.7 ± 13.6[b]	*101.3 ± 5.1*[b]	101.3 ± 9.5[b]	*85.1 (25)*	113.8 (50)
High	100	250	*101.3 ± 11.8*[b]	99.6 ± 4.5[b]	*100.2 ± 9.4*[b]	100.5 ± 14.5[b]	*92.9 (100)*	119.7 (500)

For whole blood accuracy and precision it was used four concentrations because of the wide calibration range for CsA, AM1, AM9 and AM1c.
[a] The values in parentheses are in ng/mL.
[b] The accuracy of the metabolites AM19, AM1c9 and AM4N was determined after known, but not certified concentration of pure metabolite.

Fig. 2A shows an ion extracted chromatogram of CsA and the metabolites of a blank patient sample and a random patient sample, respectively (2C) from the clinical study. No interfering peaks were observed in the blank sample. Segment 1 and 2 show the need for chromatographic separation: although fragmentation patterns are analyte specific, daughter ions with similar *m/z* values were detected for different metabolites. This is seen in segment 1, where both AM19 and AM1c9 produce peaks in both metabolite configurations. The same is seen in the AM1c configuration (5th chromatogram from the top), in which both AM1 and AM9 produce peaks in the AM1c configuration.

The average intracellular concentration of CsA per million lymphocyte of six stable renal transplant patients up to 1 month after transplantation is shown in Fig. 4. The present method has been developed in order to elucidate on the intracellular levels in patients experiencing acute rejection episodes. This will be performed prospectively in a cohort of solid transplant recipients, comparing stable patients with those experiencing rejections. The aim is to investigate if intracellular concentrations correlate better with the effect of CsA than whole blood concentrations, which is the gold standard today. An average 12-h pharmacokinetic profile of six patients with CsA, AM9 and AM19 concentrations are displayed in Fig. 5. Only two metabolites are displayed to show the lag time between CsA and formation of the primary metabolite AM9, and the lag time for the formation of the secondary metabolite AM19.

4. Conclusion

A unique specific method has been developed for the measurement of CsA and its six main metabolites intracellularly in T-lymphocytes as well as in whole blood. The method is highly specific towards CsA and its metabolites, and shows no interference with other drugs. Determinations of lymphocyte-associated

concentration in previous work have shown interesting results when correlating the lymphocyte concentration to clinical effect. However, this previously used method utilizes a less specific lymphocyte separation method, and does not allow pure T-lymphocytes concentration determination. The method described in the present paper is in addition to the more specific T-lymphocyte separation technique capable of measuring CsA's six main metabolites, which for example makes it possible to study the aspect of potential toxic metabolites.

The method is validated in accordance to guidelines from FDA. Precision, accuracy, stability, selectivity and recovery were all within the limit of acceptance. The method will be applied for explorative studies in solid organ transplant recipients. The aim is to measure intracellular and whole blood CsA and metabolite concentrations prospective in the early phase after surgery, as well as performing pharmacokinetic profiling in selected populations in order to elucidate on the effects of acute rejection episodes and toxicity.

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