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A liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for monitoring drug exposure in hematopoietic stem cell transplant recipients

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ABSTRACT

A liquid chromatography–tandem mass spectrometry method was developed for the quantification of circulating levels of multiple immunosuppressant drugs including cyclosporine (CsA), tacrolimus, methotrexate (Mtx), prednisone, prednisolone, methylprednisone, total and free mycophenolic acid (MPA), as well as MPA phenolic (MPAG) and acyl (AcMPAG) glucuronide metabolites. Linearity, precision and accuracy were validated within the typical therapeutic range of concentrations for each compound. The assay was linear over 0.125–25 ng/mL for tacrolimus, 1–500 ng/mL for prednisone/methylprednisone, 2–400 ng/mL for Mtx, 2–1000 ng/mL for prednisolone and from 7.5 to 1500 ng/mL for CsA with the lowest limit of quantification (LLOQ) being 0.125, 1.00, 2.00, 2.00 and 7.5 ng/mL, respectively. The calibration curve concentrations for MPA and MPAG ranged from 50 to 50,000 ng/mL (LLOQ: 50 ng/mL) and 10 to 10,000 ng/mL (LLOQ: 10 ng/mL) for AcMPAG. Mean recoveries in blood and plasma were 84% ± 5.7%. The method could measure individual drugs with high sensitivity, accuracy (bias ≤ 14%), and reproducibility (CV ≤ 12.8%). Its clinical application was validated by measuring levels of these drugs in samples obtained from hematopoietic stem cell transplant recipients treated with combined immunosuppressive drug therapy. Our results indicate that this approach is suitable for simultaneous determination of in vivo levels of immunosuppressive drugs commonly used in combined therapies.

1. Introduction

The use of combinations of immunosuppressant drugs is considered the therapeutic gold standard for post-allogeneic hematopoietic stem cell transplantation (HSCT) to prevent serious complications such as graft versus host disease and rejection. However, these drugs have a narrow therapeutic index and wide inter-individual pharmacokinetic fluctuations, resulting in unpredictable levels of drugs in the blood. Systemic concentrations of several immunosuppressive drugs have been correlated with their efficacy and potential life-threatening complications, supporting a need for more precise monitoring of in vivo levels of these drugs [1–3].

Immunological-based techniques are used in clinical laboratories to assess the levels of some immunosuppressant drugs in transplant recipients. However, these methods can overestimate drug concentrations, as observed for tacrolimus and cyclosporine (CsA), owing to cross-reactivity with other drugs and/or chemical moieties on biomolecules [4–6]. Therefore, a non-immunological method that could simultaneously measure immunosuppressive drugs in blood or plasma would be particularly useful. Some chromatographic techniques have been proposed for the simultaneous monitoring of multiple immunosuppressive drugs [7–10], but to our knowledge none offers the possibility of measuring CsA, tacrolimus, methotrexate (Mtx), prednisone, prednisolone, and methylprednisone in addition to total and free mycophenolic acid (MPA), as well as MPA phenolic (MPAG) and acyl (AcMPAG) glucuronide metabolites. To address this critical deficiency, we developed and validated a sensitive, specific, and accurate LC–MS/MS assay that incorporates internal standards to directly quantify these immunosuppressants in human samples.

Abbreviations: MMF, mycophenolate mofetil; CsA, cyclosporin A; Mtx, methotrexate; -G, glucuronide; HSCT, hematopoietic stem cell transplantation; LC–MS/MS, high-performance liquid chromatography–tandem mass spectrometry.

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The method was further evaluated by measuring levels of common drug combinations in HSCT patients.

2. Experimental

2.1. Chemicals

Methanol, hydrochloric acid, acetic acid and formic acid were purchased from VWR (Montréal, Qc, Canada). Ammonium formate and ammonium acetate were purchased from Laboratoire Mat (Québec, Qc, Canada). Zinc sulfate, CsA, tacrolimus, MPA, indomethacin and 6-methylprednisolone were purchased from Sigma–Aldrich Canada (Mississauga, On, Canada). Mtx, Mtx d3, AcMPAG d3, and CsA-d4 were purchased from Toronto Research Chemical Inc. (Toronto, On, Canada). MPAG and AcMPAG were supplied by Roche (Toronto, On, Canada). Prednisone and prednisolone were purchased from Steraloids (Newport, RI, USA) and prednisolone d6 from C/D/N Isotopes (Montréal, Qc, Canada). Strata™-X Reversed SPE Phase Sorbents were purchased from Phenomenex (Torrance, CA, USA) and Centrifree tubes were obtained from Millipore Corporation (Bedford, MA, USA).

2.2. Blood samples

Human blood was collected from 4 males and 2 females transplant recipients who participated in a prospective HSCT study, which was approved by the Institutional Review Board of our institution. All participants, age between 36 and 57 years old, received hematopoietic stem cells to treat a malignant hematological disease. The source of the stem cells was from mobilized peripheral blood progenitor cells from related donors, except for one individual transplanted with unrelated umbilical cord blood. After transplantation, patients received a prophylactic immunosuppressive regimen as determined by the physician who performed the transplant. Each patient received the following drugs, CsA or tacrolimus along with Mtx or MPA, with or without methylprednisolone, such that each patient received at least two drugs in total except for one individual who received only tacrolimus. Briefly, patients under Mtx therapy received an intravenous bolus of 15 mg/m^2 day 1 and 10 mg/m^2 days 3, 6 and 11. The doses at days 3, 6, and 11 were adjusted downwards in case of renal and hepatic dysfunction or other serious adverse effects. Methylprednisolone was used at 1 mg/kg/dose intravenously. Depending on the transplant procedure, an oral relay was thereafter adjusted to reach a trough blood level between 200 and 400 ng/mL. Tacrolimus was adjusted to maintain trough blood concentration from 5 to 20 ng/mL. MMF was used at fixed dosing regimens of 15 mg/kg orally twice daily.

Blood samples were collected 2 h after an intravenous administration of Mtx at day 3, 6 and 11 post-transplantation. For methylprednisolone, plasma concentration was determined 2 h after intravenous drug administration. Tacrolimus and CsA were assessed in blood samples collected prior to oral drug intake (C_0, or drug concentration at equilibrium) and 2 h after drug administration (C_2). Samples from patients under oral MMF therapy were collected at C_0, C_2, C_4, and C_6 after drug intake.

Venous blood samples (6 mL) were collected in EDTA (K2)-containing Vacutainer tubes from a peripheral catheter and immediately placed on ice. A 500 μL aliquot of blood was frozen at −80 °C until analysis of CsA/tacrolimus levels. The remaining blood was centrifuged at 2000 × *g* at 4 °C for 10 min to isolate plasma, which was divided into three aliquots. One aliquot of 500 μL was acidified with 8 μL of 85% phosphoric acid and used to measure other drugs including total MMF metabolites. The remaining aliquot was non-acidified and used to measure free (unbound) MPA as described [11]. Plasma samples were stored at −80 °C.

2.3. Sample preparation

2.3.1. Stock solutions, working solutions, calibration standards and quality control samples

Stock solutions of CsA (300 μg/mL), CsA d4 (100 μg/mL), tacrolimus (100 μg/mL), Mtx (100 μg/mL), Mtx d3 (100 μg/mL), MPA (5 mg/mL), MPAG (5 mg/mL), indomethacin (1 mg/mL), prednisolone (100 μg/mL), prednisone (100 μg/mL), methylprednisolone (100 μg/mL) and prednisolone d6 (100 μg/mL) were prepared by dissolving each compound in methanol. AcMPAG (1 mg/mL) and AcMPAG d4 (100 μg/mL) stock solutions were prepared in methanol acidified with 0.3 g/L phosphoric acid. All solutions were stored at −80 °C.

Working solutions of CsA, tacrolimus and CsA d4 were prepared by diluting each stock solution in an appropriate volume of HPLC-grade methanol, and MPA, MPAG, AcMPAG, prednisolone, prednisone, methylprednisolone and Mtx were prepared in acidified methanol and stored at −20 °C (Supplementary Table 1). The internal standards indomethacin, AcMPAG d3, prednisolone d6 and Mtx d3 were prepared in acidified methanol and kept at 4 °C.

Calibration standards were prepared by diluting 25 μL of each working solution with 0.475 mL of appropriate matrix (human blood, acidified plasma or NaCl) to achieve a specific concentration needed for the calibration (Supplementary Table 2). Quality control (QC) samples at low, medium, and high concentrations were prepared in glass tubes by diluting stock solutions in appropriate matrix and storing at −80 °C.

2.3.2. Sample preparation

Blood sample preparation for the analysis of CsA and tacrolimus was based on the procedure described by Bogusz et al. [10]. Briefly, in a test tube, 100 μL of blood, 250 μL of water, 250 μL of 0.1 M zinc sulfate, and 500 μL of an internal standard solutions were added and thoroughly mixed by vortexing for 30 s; the mixture was then left for 10 min at room temperature and then centrifuged at ∼2400 × *g* for 10 min. Clear supernatant was collected, and 2 mL of 0.1 M HCl was added prior to solid-phase extraction.

Solid-phase extraction followed our method [11] with some modifications. Mtx, prednisone, prednisolone, methylprednisone and total MMF metabolites, were extracted using acidified plasma (see Section 2.2). The free MPA concentrations were determined by using non-acidified plasma samples applied to Centrifree tubes (Millipore Corporation, Bedford, MA) and centrifuged at 4500 × *g* for 20 min at 20 °C. Briefly, 100 μL of sample was mixed with 2 mL of 0.1 M HCl and 50 μL of standard solution. Samples were thoroughly mixed by vortexing for 30 s and then applied to a Strata-X 60 mg (Phenomenex, Torrance, CA, USA) cartridge previously conditioned with 1 mL methanol followed by 2 mL of 0.1 M HCl. The loaded cartridges were then sequentially washed with 2 mL water and 2 mL of 25% methanol, dried under vacuum, and the analytes eluted with 2 mL of methanol. Prior to analysis, methanol was evaporated under nitrogen at 20 °C for 30 min with a turbo Vap system (Zymark Corporation, Hopkinton, MA, USA). The residue was dissolved in 100 μL of 50% methanol containing 1 mM ammonium formate and 0.1% formic acid.

2.4. High-performance liquid chromatography conditions

The chromatographic system consisted of an UFLC Prominence (Shimadzu Scientific Instruments Inc., Columbia, MD, USA) coupled to an API4000 mass spectrometer (AB Sciex, Concord, On, Canada). The MS was operated in multiple reactions monitoring mode and equipped with a turbo ion-spray source. Electrospray ionization was performed in positive ion mode. The voltage was held at 5500 V. The resolution used in those methods for Q1 and Q3 was Unit/Unit. Declustering potential and collision energy, ion

Table 1
Optimized detection parameters and LC–MS/MS conditions.

Analytes	MRM transition (*m*/*z*)	DP (V)	CE (V)	Source temperature (°C)	LLOQ (ng/mL)	Retention time (min)
CsA	1219.9 → 1203.0	66	27	250	7.5	2.4
Tacrolimus	821.5 → 768.6	71	39	250	0.125	2.1
CsA d4	1224.0 → 1207.0	66	27	250	–	2.4
Mtx	455.2 → 308.1	71	29	650	2	1.7
Mtx d3	458.2 → 311.1	71	29	650	–	1.7
MPA	321.0 → 207.0	71	56	650	50	7.5
MPAG	514.3 → 321.0	46	35	650	50	3.2
AcMPAG	514.3 → 321.0	46	35	650	10	4.6
AcMPAG d3	517.3 → 324.0	46	35	650	–	4.6
Indomethacin	358.2 → 138.8	50	52	650	–	10.1
Prednisone	359.2 → 341.2	61	19	650	1	4.2
Prednisolone	361.3 → 343.1	51	9	650	2	4.4
Methylprednisolone	375.1 → 357.1	56	28	650	1	6.1
Prednisolone d6	367.3 → 349.1	51	9	650	–	4.4

source temperature setting, and mass transitions (*m*/*z*) for detection are listed in Table 1. The system was controlled through Analyst Software, version 1.5.

For MPA, MPAG, AcMPAG, Mtx, prednisone, prednisolone and methylprednisolone, the chromatographic separation was achieved with an ACE-3 HL C_{18} column containing 3-μm packing material, 100 mm × 4.6 mm (Canadian Life Science, Peterborough, Canada). The mobile phases consisted of water with 3 mM ammonium formate and 0.1% formic acid (solvent A), and methanol with 3 mM ammonium formate and 0.1% formic acid (solvent B). The flow rate was 0.9 mL/min. The analytes were eluted using the following program: 0–6 min, linear gradient 50–65% B; 6–6.1 min, linear gradient 65–85%; 6.1–8 min, isocratic 85% B; 8–8.1 min, linear gradient 85–95% B; 8.1–11 min, isocratic 95% B; 11–11.1 min, linear gradient 95–50% B; 11.1–14 min, isocratic 50% B. For tacrolimus and CsA, the chromatographic separation was achieved with a Luna C8 containing 5-μm packing material, 50 mm × 4.6 mm (Phenomenex, Torrance, CA, USA). The mobile phase consisted of water with 10 mM ammonium acetate and 0.1% acetic acid (solvent A), and methanol with 10 mM ammonium acetate and 0.1% acetic acid (solvent B). The flow rate was 0.9 mL/min. The analytes were eluted using the following program: 0–0.5 min, linear gradient 50–97% B; 0.5–2.5 min, isocratic 97% B; 2.5–2.6 min, linear gradient 97–50% B; 2.6–5.5 min, 50% B.

2.5. Data analysis

For patients receiving MPA, area under the concentration-time curve from 0 to 6 h ($AUC_{0-6\,h}$) was calculated using the linear trapezoidal method using WinNoLin v5.01 software (Pharsight, Mountain View, CA, USA). All other calculations were performed with Microsoft® Office Excel 2007 using standard formula functions. Values are expressed as mean and standard deviation or percentage.

2.6. Method validation

The intra- and inter-day precision is defined as the coefficient of variation (CV, %), whereas the accuracy (bias, %) is determined as follows: [(measured QC concentration – reference QC concentration)/reference QC concentration] × 100. The intra- and inter-day validation was performed by analyzing three replicates of QC samples on three different days. The recovery after the extraction procedure was determined by comparing the peak areas of QC samples spiked prior to and after extraction. Results are expressed as a percentage of the area of the extracted QC relative to the directly injected reference standard.

A seven-point calibration curve was prepared by spiking plasma or whole blood with the appropriate amount of each analyte. The linear regression of MPA/indomethacin, MPAG/AcMPAG d3, AcMPAG/AcMPAG d3, prednisone/prednisolone d6, prednisolone/prednisolone d6, methylprednisolone/prednisolone d6, Mtx/Mtx d3, tacrolimus/CsA d4 and CsA/CsA d4 peak area ratios was weighted by $1/x^2$. The coefficient of determination (R) was used to evaluate the linearity of the calibration curve. The lower limit of quantification (LLOQ) was defined as the minimum value at which the ratio of signal-to-noise was ≥5:1.

Short- and long-term stabilities of the analytes were investigated at QC low and high concentrations by analyzing samples stored at 20 °C for 4 h or at −80 °C for 4 or 9 months in blood and plasma. To evaluate freeze/thaw stability, QC samples were subjected to freezing for at least 24 h at −80 °C and thawed unassisted at room temperature (1 h) for three cycles. Stability of the processed samples in the reconstitution solution was assessed by keeping extracted QC samples at 4 °C for 24 h and then quantifying and comparing the concentration with freshly extracted samples. Stock solution stability was investigated for solutions of analytes after storage at −80 °C. All stability evaluations were performed in triplicate.

3. Results and discussion

The method outlined in this work allows the monitoring of drugs used in combined immunosuppressants therapy in a single blood sample (Supplementary Fig. 1), namely CsA, tacrolimus, Mtx, prednisone, prednisolone and methylprednisone, MPA and MPA metabolites (MPAG, AcMPAG). Plasma was used to measure all the drugs except for CsA and tacrolimus, for which whole blood was used. This approach was justified based on two main reasons. First, in the clinical setting the appropriate therapeutic range to maximize the effectiveness of CsA and tacrolimus is currently determined using the total level of these drugs in whole blood. Second, CsA and tacrolimus are preferentially distributed in erythrocytes with a blood:plasma ratio of approximately 2 for CsA and more than 10 for tacrolimus [12–15]. Thus, their concentrations in the plasma fraction were underestimated in comparison to the total concentration in whole blood (data not shown).

3.1. Selectivity

In the validation process, we first addressed whether the chromatographic method was selective for the targeted drugs. Representative chromatograms for blood and plasma samples are

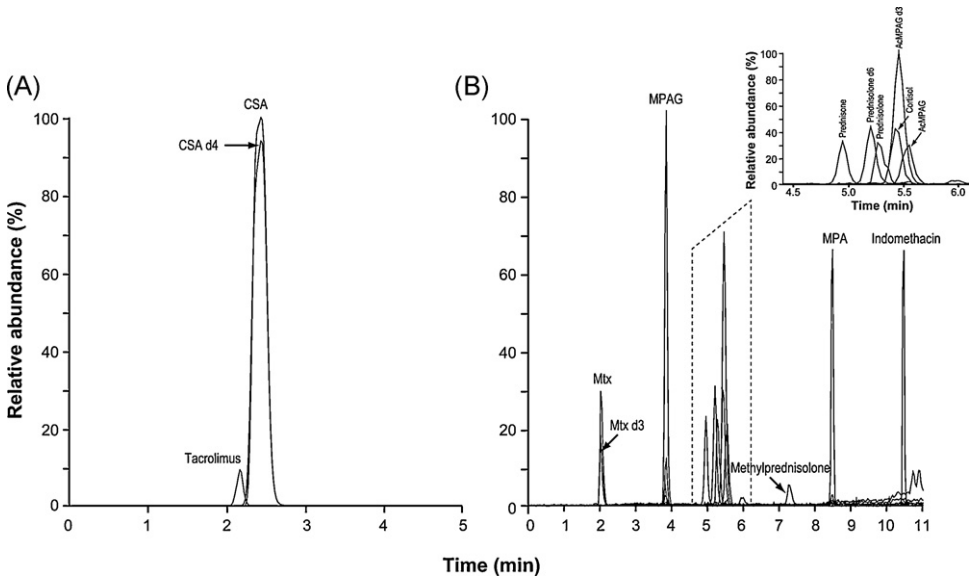

Fig. 1. Chromatographic separation of (A) CsA, tacrolimus and CsA d4, and (B) Mtx, MPA, MPAG, AcMPAG, prednisone, prednisolone, methylprednisone, cortisol and internal standards (AcMPAG d3, indomethacin, Mtx d3, and prednisolone d6).

presented in Fig. 1A and B, respectively; each drug was resolved with baseline separation. Selectivity was confirmed by evaluating the signal in the blank matrix and by mixing each drug in the appropriate matrix (Supplementary Table 2). No additional peak due to endogenous substances was observed (Figs. 2 and 3). Furthermore, the presence of endogenous cortisol in plasma is a major concern considering its potential interference with prednisolone quantification (i.e., these steroids differ by only 2 atomic mass units) [16]. Consequently, we assessed the ability of the method to distinguish cortisol from other corticosteroids by adding cortisol into a working solution. As shown in Fig. 1B, cortisol could indeed be separated from other compounds.

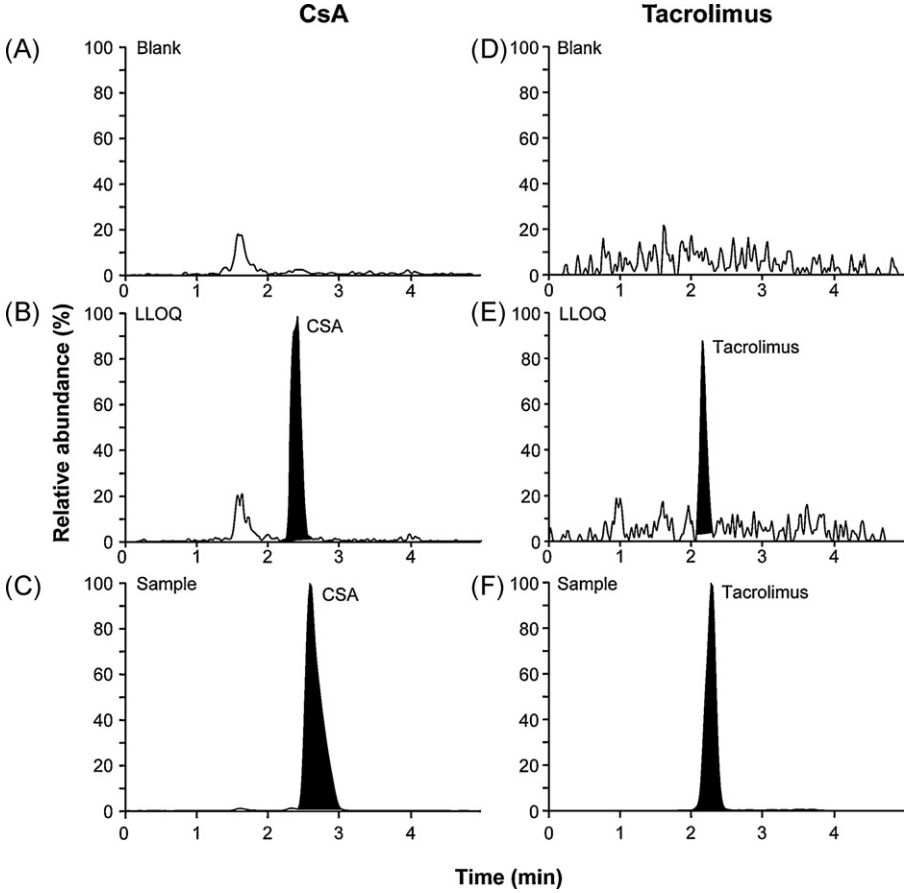

Fig. 2. Chromatograms of CsA (A–C) and tacrolimus (D–F) in blood. For each analyte, a blank (A and D), the LLOQ (B and E), and a patient sample (C and F) are represented. CsA and tacrolimus concentrations in the sample were 273.0 ng/mL (C) and 15.7 ng/mL (F).

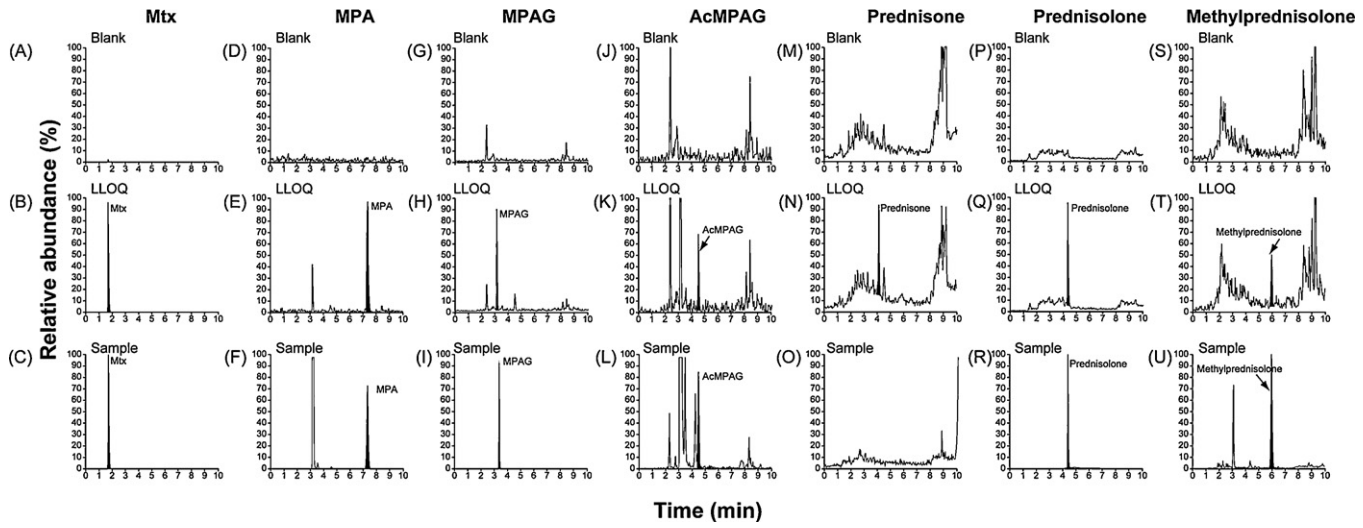

Fig. 3. Chromatograms of Mtx (A–C), MPA (D–F), MPAG (G–I), AcMPAG (J–L), prednisone (M–O), prednisolone (P–R) and methylprednisolone (S–U) in plasma. For each analyte, a blank, the LLOQ, and a patient sample are represented. Concentrations of Mtx, MPA, MPAG, AcMPAG, prednisolone and methylprednisolone in the sample were 478, 3140, 7000, 312, 189 and 132 ng/mL, respectively. None of the volunteers were taking prednisone. The chromatogram O represents analysis of a plasma sample from a patient receiving tacrolimus as single immunotherapy. The absence of resolved peak with baseline separation at the retention time of prednisone (4.4 min) confirms the absence of this drug in the sample.

As previously reported [7] MPA glucuronide metabolites produced, upon in-source fragmentation, an identical precursor product ion pair for MPA (see MRM transition mass at Table 1) that could interfere with the MPA quantification by co-elution. However, this drawback of measurement of MPAG and AcMPAG by mass spectrometry is overcome by an adequate chromatographic separation. As shown in Fig. 1B, MPA, MPAG and AcMPAG are sufficiently separated and displayed a distinct retention time.

3.2. Recovery and suppression ionization

It is well established that endogenous components in a specific matrix can alter ionization efficiency and affect the precision and accuracy of an analytical method [17,18]. To evaluate the potential influence of a given matrix on MS measurements, we performed a standard post-extraction spike method or ionization suppression test. Briefly, this consists of comparing analyte response in neat solution against analyte reconstituted in neat solution and added to an extracted matrix blank [19,20]. As confirmed by the suppression ionization percentage assessed at high concentration, matrix had the greatest impact on ionization efficacy for CsA (−28.0%) and Mtx (−22.2%) (Table 2). As supported by a low CV (≤12.8%) for intra- and inter-day precision validation analyses (Table 3), the matrix effect did not appear to affect the assay performance. Also, the use of internal standards accounts for such potential variability and ensures the accuracy of measurements [21]. The extraction efficiency is also depicted in Table 2. Overall recovery was determined for low and high concentrations of each drug.

3.3. Linearity and lower limit of quantification

The linearity of quantification in plasma was in the range of 50–50,000 ng/mL for MPA and MPAG (R: 0.996 and 0.997, respectively), 10–10,000 ng/mL for AcMPAG (R: 0.999), 2–400 ng/mL for Mtx (R: 0.993), 1–500 ng/mL for prednisone and methylprednisolone (R: 0.999 and R: 0.992, respectively), 2–1000 ng/mL for prednisolone (R: 0.997), and in blood from 7.5 to 1500 ng/mL for CsA (R: 0.997), and 0.125–25 ng/mL for tacrolimus (R: 0.996). LLOQ values for the compounds are presented in Table 1. Linearity was observed over a wide range of concentrations for each drug and thus allowed the quantification of these immunosuppressive drugs ranging from nanogram per milliliter for tacrolimus and corticosteroids to milligram per milliliter for MPA and CSA.

3.4. Precision, accuracy and stability

The intra- and inter-day precision (CV, %) and accuracy (bias, %) values for each drug are listed in Table 3. CVs were equal to or less than 12.8%, and bias ranged from −12.6 to 14.0. Short- (4 h at 20 °C) and long-term stability (4–9 months at −80 °C), freeze/thaw stability, and stability of the processed samples left in the autosampler (24 h at 4 °C) are shown in Table 2. Stability values are

Table 2
Stability of the analytes and extraction recovery.

	Blood				Plasma							
	CsA		Tacrolimus		Mtx		Prednisone		Prednisolone		Methylprednisolone	
	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High
Concentration (ng/mL)	22.5	1200.0	0.4	20.0	6.0	320.0	3.0	400.0	6.0	800.0	3	400.0
Stability (bias, %)												
Short term	8.5	−1.4	−6.4	−9.5	−1.7	4.4	−6.0	2.0	−10.2	−8.8	−9.23	1.7
Long term	0.6	−1.5	4.8	12.4	14.4	12.8	−10.3	−8.6	−3.2	−14.8	3.5	−7.1
Freeze/thaw	1.2	−0.5	11.6	7.5	0.1	11.7	−10.9	0.3	−8.6	−9.7	−12.7	−1.2
In the autosampler	−7.0	−1.1	5.8	3.6	8.7	11.0	−8.9	1.1	−3.4	−6.3	−16.4	0.1
Recovery (%)	86.5	93.6	79.7	88.7	83.1	80.0	89.8	89.3	89.9	91.8	70.2	88.0
Suppression ionization	–	−28.0	–	−12.9	–	−22.2	–	−10.8	–	−8.4	–	−5.9

Table 3
Assay precision.

	CsA			Tacrolimus		
	Low	Med	High	Low	Med	High
Blood						
QC concentrations (ng/mL)	22.5	750.0	1200.0	0.4	12.5	20.0
Intra-day						
Average (ng/mL)	23.0	784.6	1200.9	0.3	11.7	18.8
CV (%)	2.7	1.8	2.0	7.2	12.8	8.0
Bias (%)	2.2	4.6	0.2	−8.6	−6.2	−6.0
Inter-day						
Average (ng/mL)	22.2	762.1	1175.5	0.3	12.2	19.4
CV (%)	5.5	4.8	5.2	8.0	11.5	8.5
Bias (%)	−1.5	1.6	−2.0	−7.2	−2.7	−3.0

	Mtx			Prednisone			Prednisolone			Methylprednisone		
	Low	Med	High	Low	Med	High	Low	Med	High	Low	Med	High
Plasma												
QC concentrations (ng/mL)	6.0	200.0	320.0	3.0	250.0	400.0	6.0	500.0	800.0	3.0	250.0	400.0
Intra-day												
Average (ng/mL)	5.9	214.4	336.7	2.7	3.1	394.3	5.9	514.3	791.5	2.6	243.9	389.2
CV (%)	7.9	1.4	3.9	2.9	3.1	4.4	3.5	1.3	3.5	4.1	2.7	3.4
Bias (%)	−1.3	7.2	5.2	−10.4	−2.2	−1.4	−1.2	2.9	−1.1	−12.6	−2.4	−2.7
Inter-day												
Average (ng/Ml)	6.0	228.1	353.0	2.8	265.6	430.7	5.8	508.1	771.8	2.9	265.0	426.4
CV (%)	6.7	7.3	4.4	5.7	5.8	6.2	6.0	6.3	7.1	8.0	7.3	7.7
Bias (%)	0.6	14.0	10.3	−7.3	6.2	7.7	−2.7	1.6	−3.5	−3.8	6.0	6.6

expressed as the bias (%) compared with the initial concentration and was assessed in blood for CsA and tacrolimus and in plasma for other drugs. Stock solutions were stable for at least 1 year at −20 °C, with measured concentrations varying by <5% from those measured when the stock solutions were initially prepared.

3.5. Application of the analytic method

Therapeutic drug monitoring for CsA and tacrolimus is routinely performed in the clinic to ensure drug efficacy and to limit toxicity. The EMIT immunoassay is an analytic method frequently used for therapeutic drug monitoring in clinical setting [22], and does not involve sophisticated instruments but is far less specific and sensitive than the method presented here. Indeed, the concentration of drug can be overestimated with the EMIT method owing to immunological cross-reactivity with other drugs and their metabolites, or with endogenous antibodies in the sample [4–6]. Also, MPA glucuronidated metabolites, some of which is suspected to be toxic (AcMPAG), cannot be quantified with this method [23]. MPA and its metabolites can indeed be measured using HPLC coupled with ultraviolet detection [24], but ultraviolet detection is less specific and sensitive than MS/MS. Furthermore, these techniques are not capable of measuring free MPA [25,26].

Several analytic methods have been developed for simultaneous quantification of various immunosuppressant drugs [7,8,11,27,28]; to our knowledge, however, none of them have been validated for simultaneous measurement of CsA, tacrolimus, Mtx, various corticosteroids, total and free MPA and its glucuronide metabolites, all of which are commonly used in combined regimens prescribed in HSCT. The suitability of our method was demonstrated by testing clinical samples collected from six HSCT recipients (Table 4). C_0 values for tacrolimus and CsA assessed by an immunoassay technique (CEDIA Cyclosporine Plus, Emit *2000* Tacrolimus Assay)

Table 4
Pharmacokinetic parameters for HSCT patients treated with immunosuppressive drugs.

Drug	Drug dosage	Sampling	Drug concentrations
CsA	1.9 mg/kg/day	C_0	273.0 ng/mL
		C_2	872.0 ng/mL
Tacrolimus	0.15 mg/kg/day	C_0	15.7 ng/mL
		C_2	43.8 ng/mL
Mtx	17.5 ± 0.0 mg I.V.[a]	C_2	362.3 ± 49.9 ng/mL[a]
	16.7 ± 5.8 mg I.V.[a]	C_2	436.7 ± 115.8 ng/mL[a]
	19.3 ± 0.6 mg I.V.[a]	C_2	546.0 ± 96.2 ng/mL[a,b]
Mycophenolate mofetil	1000 mg twice daily	AUC_{0-6} MPA	12.5 mg h/L
		AUC_{0-6} MPAG	451.0 mg h/L
		AUC_{0-6} AcMPAG	1.5 mg h/L
	1250 mg twice daily	AUC_{0-6} MPA	6.7 mg h/L
		AUC_{0-6} MPAG	940.0 mg h/L
		AUC_{0-6} AcMPAG	4.5 mg h/L
Methylprednisone	50 mg I.V.		62.8 ng/mL

Of the six patients tested, one received CsA, one tacrolimus, three Mtx, two MMF and one methylprednisone.
Area under the concentration-time curve from 0 to 6 h ($AUC_{0-6\,h}$) is expressed as mg h/L.
C_0, drug concentration measured before drug intake at equilibrium; C_2, drug concentration measured 120 min after drug administration; I.V., intravenous bolus.
[a] The cumulative mean values ± SD for days 3, 6 and 11 post-transplantation.
[b] A blood sample was missing at day 3 for one patient.

at the recruiting hospital center were highly similar to those measured with our method (tacrolimus: 14.7 ng/mL and 15.7 ng/mL; CsA: 317 ng/mL and 273 ng/mL, respectively). Tacrolimus and CsA concentrations measured at C_0 and C_2 were consistent with values previously reported for similar doses given to patients receiving a solid-organ transplant [29,30]. Plasma levels of Mtx at C_2 were highly similar across patients, and to our knowledge these measurements have never been reported in a clinical context. The mean dose and mean plasma concentration of Mtx were 17.8 mg/patient (intravenous) and 436.1 ± 107.9 ng/mL, respectively. The results for MPA are in agreement with the $AUC_{0-6\,h}$ values reported for HSCT or solid-organ transplants; moreover, as demonstrated in healthy volunteers and transplant recipients, the AUC values for MPA and its glucuronide metabolites are highly variable [11,31,32]. $AUC_{0-6\,h}$ values in patients varied by 97%, 50% and 70% for MPA, MPAG and AcMPAG, respectively.

4. Conclusion

We report a highly sensitive and specific LC–MS/MS method, allowing for the first time the simultaneous monitoring and quantification of several common immunosuppressive drugs. The robustness of the method was demonstrated and linearity was validated for a large range of concentrations including the therapeutic range. This method requires only a small-volume peripheral blood sample, an attribute that may be beneficial to HSCT patients in the clinic. Indeed, owing to the frequent administration of myeloablative conditioning regimens in the pre-transplantation period, the recovery of hematologic function is slow and gradual after engraftment. It is therefore favorable to limit blood sample collection in the immediate post-transplantation period. Also, this method is convenient for large-scale clinical studies aimed at improving and optimizing patient outcomes. It could also be useful in therapeutic drug monitoring to promote the efficient use of immunosuppressive drugs and thus to limit life-threatening complications related to non-optimal exposure to immunosuppressive medications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.12.029.

References

[1] M. Oellerich, V.W. Armstrong, Ther. Drug Monit. 28 (2006) 720.
[2] P.F. Halloran, N. Engl. J. Med. 351 (2004) 2715.
[3] B. Bolwell, R. Sobecks, B. Pohlman, S. Andresen, L. Rybicki, E. Kuczkowski, M. Kalaycio, Bone Marrow Transplant. 34 (2004) 621.
[4] W. Steimer, Clin. Chem. 45 (1999) 371.
[5] J.M. Tredger, N. Roberts, R. Sherwood, G. Higgins, J. Keating, Clin. Chem. Lab. Med. 38 (2000) 1205.
[6] J. Hermida, J.C. Tutor, Ther. Drug Monit. 31 (2009) 269.
[7] R. Difrancesco, V. Frerichs, J. Donnelly, C. Hagler, J. Hochreiter, K.M. Tornatore, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 859 (2007) 42.
[8] R.A. Koster, E.C. Dijkers, D.R. Uges, Ther. Drug Monit. 31 (2009) 116.
[9] C. Seger, K. Tentschert, W. Stoggl, A. Griesmacher, S.L. Ramsay, Nat. Protoc. 4 (2009) 526.
[10] M.J. Bogusz, E.A. Enazi, H. Hassan, J. Abdel-Jawaad, J.A. Ruwaily, M.A. Tufail, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 850 (2007) 471.
[11] M.O. Benoit-Biancamano, P. Caron, E. Levesque, R. Delage, F. Couture, C. Guillemette, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 858 (2007) 159.
[12] R.J. Ptachcinski, R. Venkataramanan, G.J. Burckart, Clin. Pharmacokinet. 11 (1986) 107.
[13] F. Akhlaghi, A.K. Trull, Clin. Pharmacokinet. 41 (2002) 615.
[14] L. Backman, M. Nicar, M. Levy, D. Distant, C. Eisenstein, T. Renard, R. Goldstein, B. Husberg, T.A. Gonwa, G. Klintmalm, Transplantation 57 (1994) 519.
[15] W.J. Jusko, R. D'Ambrosio, Transplant. Proc. 23 (1991) 2732.
[16] V.A. Frerichs, K.M. Tornatore, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 802 (2004) 329.
[17] N.R. Srinivas, Biomed. Chromatogr. 23 (2009) 451.
[18] P.J. Taylor, Clin. Biochem. 38 (2005) 328.
[19] C. Cote, A. Bergeron, J.N. Mess, M. Furtado, F. Garofolo, Bioanalysis 1 (2009) 1243.
[20] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[21] N.T. Vethe, L.C. Gjerdalen, S. Bergan, Scand. J. Clin. Lab. Invest. 70 (2010) 583.
[22] R.G. Morris, Ann. Pharmacother. 39 (2005) 119.
[23] M. Shipkova, E. Schutz, I. Besenthal, P. Fraunberger, E. Wieland, Ther. Drug Monit. 32 (2010) 79.
[24] C.G. Patel, F. Akhlaghi, Ther. Drug Monit. 28 (2006) 116.
[25] B. Atcheson, P.J. Taylor, D.W. Mudge, D.W. Johnson, P.I. Pillans, S.E. Tett, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 799 (2004) 157.
[26] C.G. Patel, A.E. Mendonza, F. Akhlaghi, O. Majid, A.K. Trull, T. Lee, D.W. Holt, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 813 (2004) 287.
[27] P. Guo, X. Wang, L. Liu, M.G. Belinsky, G.D. Kruh, J.M. Gallo, J. Pharm. Biomed. Anal. 43 (2007) 1789.
[28] B.C. McWhinney, S.E. Briscoe, J.P. Ungerer, C.J. Pretorius, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 878 (2010) 2863.
[29] F. Citterio, Transplant. Proc. 36 (2004) 420S.
[30] C. Dansirikul, C.E. Staatz, S.B. Duffull, P.J. Taylor, S.V. Lynch, S.E. Tett, Ther. Drug Monit. 26 (2004) 593.
[31] A. Okamura, M. Yamamori, M. Shimoyama, Y. Kawano, H. Kawano, Y. Kawamori, S. Nishikawa, K. Minagawa, K. Yakushijin, Y. Katayama, T. Sakaeda, M. Hirai, T. Matsui, Int. J. Hematol. 88 (2008) 104.
[32] P. Jacobson, S.F. El-Massah, J. Rogosheske, A. Kerr, J. Long-Boyle, T. DeFor, C. Jennissen, C. Brunstein, J. Wagner, M. Tomblyn, D. Weisdorf, Bone Marrow Transplant. 44 (2009) 113.