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# Sensitive high-performance liquid chromatography–tandem mass spectrometry method for quantitative analysis of mycophenolic acid and its glucuronide metabolites in human plasma and urine

Marie-Odile Benoit-Biancamano [a], Patrick Caron [a], Éric Lévesque [a,b], Robert Delage [c], Félix Couture [b], Chantal Guillemette [a,*]

[a] *Canada Research Chair in Pharmacogenomics, Laboratory of Pharmacogenomics, Oncology and Molecular Endocrinology Research Center, CHUL Research Center and Faculty of Pharmacy, Laval University, G1V 4G2 Québec, Canada*
[b] *Department of Hematology and Oncology, Hôtel-Dieu de Québec Hospital, Faculty of Medicine, Laval University, Québec, Canada*
[c] *Department of Hematology, Enfant-Jésus Hospital, Faculty of Medicine, Laval University, Québec, Canada*

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**Abstract**

A method to determine total and free mycophenolic acid (MPA) and its metabolites, the phenolic (MPAG) and acyl (AcMPAG) glucuronides, using HPLC and mass spectrometry was developed. Mean recoveries in plasma and urine samples were >85%, and the lower limits of quantification for MPA, MPAG and AcMPAG were 0.05, 0.05 and 0.01 mg/L, respectively. For plasma, the assay was linear over 0.05–50 mg/L for MPA and MPAG, and from 0.01 to 10 mg/L for AcMPAG. A validation study demonstrated good inter- and intra-day precision (CV ≤ 11%) and accuracy (bias ≤ 16%) and satisfactory specificity and stability. Pharmacokinetic parameters were assessed in plasma and urine from healthy volunteers after an oral dose of mycophenolate mofetil.


*Keywords:* HPLC; Mass spectrometry; Mycophenolic acid; MPAG; AcMPAG; Glucuronide

## 1. Introduction

Mycophenolic acid (MPA) is the active immunosuppressant metabolite of the ester prodrug mycophenolate mofetil (MMF) [1]. MMF is widely used to prevent rejection in solid organ and hematopoietic cell recipients [2]. Its immunosuppressant properties are based on its selective inhibition of inosine monophosphate dehydrogenase II, a key enzyme in *de novo* synthesis of guanosine nucleotides in lymphocytes. MPA therefore leads to arrested proliferation and decreased function of T and B lymphocytes [1–3]. MMF is very rapidly and extensively (95%) converted to MPA, making its detection in plasma unfeasible after oral administration [4]. MPA is converted to its major metabolite, phenolic glucuronide (MPAG), by uridine diphosphate glucuronosyltransferase enzymes (UGTs). Two minor metabolites, 7-*O*-glucoside and acyl glucuronide (AcMPAG), have also been described [5–7]. AcMPAG is pharmacologically active [7–9] and is considered a possible initiating event for toxicity [8,10,11].

Several studies have correlated the pharmacokinetics of MPA with a risk for rejection [12,13]. Because MPA pharmacokinetics are characterized by large interindividual variability, better clinical results might be achieved with the individualization of MMF dose. Different strategies, such as pharmacokinetic and pharmacodynamic monitoring, or dose individualization based on pharmacogenetic information, have been proposed [14]. However, adequate quantification of the active metabolites MPA and AcMPAG and the inactive MPAG may be critical for therapeutic monitoring. In addition, because MPA is highly bound to plasma proteins [15], quantification of free MPA is also likely to be needed.

Several techniques have been developed to quantify MPA and its two glucuronide metabolites in plasma. High-performance liquid chromatography (HPLC) followed by ultraviolet (UV) detection is the most frequently employed technique [16–19]. The EMIT assay [20], a commercial immunoassay, is a popular alternative. A few studies also have validated the use of

* Corresponding author. Tel.: +1 418 654 2296; fax: +1 418 654 2761.
*E-mail address:* chantal.guillemette@crchul.ulaval.ca (C. Guillemette).

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HPLC–MS/MS [21–23]. However, despite the large number of methods that have been described, no single method has been validated to measure both total and free MPA as well as MPAG and AcMPAG in plasma. Moreover, no methods have been validated that measure MPA/metabolites in urine matrix.

To address the need for a more comprehensive analysis of MPA and its metabolites, we established a reliable and sensitive LC–MS/MS method to simultaneously analyze total MPA, MPAG and AcMPAG in human plasma and urine as well as to quantify free MPA in plasma.

## 2. Experimental

### 2.1. Chemicals

All chemicals were of HPLC grade. Methanol, hydrochloric acid (HCl), glacial acetic acid and phosphoric acid were obtained from VWR Canlab (Montreal, Que., Canada). MPA was purchased from Sigma Diagnostics Canada (Mississauga, ON, Canada). MPAG and AcMPAG were supplied by Roche. Indomethacin, ammonium formate, sodium chloride and $Na_2HPO_4$ were purchased from Sigma (St. Louis, MO, USA).

### 2.2. Plasma and urine samples

Human plasma and urine were collected from healthy volunteers who participated in a pharmacokinetic–pharmacogenetic study approved by the Institutional Review Board of the Centre Hospitalier de l'Université Laval, Enfant-Jésus Hospital and Hôtel-Dieu de Québec [24]. Venous blood samples (3 mL), collected in EDTA ($K_2$)-containing vacutainer tubes, were obtained from 31 male and 21 female healthy volunteers who received a single 1.5 g oral dose of MMF. Blood samples were collected before dosing and at 20 min, 40 min, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h and 12 h after MMF administration from a peripheral catheter and immediately placed on ice. Samples were centrifuged at 2000 × *g* for 10 min at 4 °C. Plasma was isolated, and three aliquots were prepared for each sample. The first aliquot (500 μL) was acidified with 8 μL of 85% phosphoric acid; two additional aliquots were prepared (500 and 100–500 μL) with the remaining plasma. Plasma aliquots were immediately placed on dry ice and then stored at −80 °C. Sample acidification was necessary to avoid degradation of AcMPAG, which is unstable at physiologic pH [21]. Urine from volunteers was collected in two fractions: between 0 and 6 h, and from 6 to 12 h after drug intake. The samples were kept on ice at all times. The total urine volume was measured, and four aliquots of 4 mL were prepared. The first two aliquots were acidified with 32 μL of 85% phosphoric acid. Aliquots were then immediately placed on dry ice and then stored at −80 °C. Plasma and urine samples also were collected from two healthy donors who did not receive the drug, and these samples were used to prepare the in-house calibration standards and quality controls.

### 2.3. Sample preparation

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

Stock solutions of MPA (5 mg/mL), MPAG (5 mg/mL) and indomethacin (1 mg/mL) were prepared by dissolving each of

Table 1
MPA, MPAG and AcMPAG working solutions and calibration standard

| Working solution concentrations (mg/L) | | | Calibration standard matrix | Calibration standard concentrations (mg/L) | | |
|---|---|---|---|---|---|---|
| MPA | MPAG | AcMPAG | | MPA | MPAG | AcMPAG |
| 200 | – | – | NaCl | 10 | – | – |
| 100 | – | – | | 5 | – | – |
| 20 | – | – | | 1 | – | – |
| 10 | – | – | | 0.5 | – | – |
| 2 | – | – | | 0.1 | – | – |
| 0.4 | – | – | | 0.02 | – | – |
| 0.2 | – | – | | 0.01 | – | – |
| 1000 | 400 | 800 | Acidified urine | 50 | 20 | 40 |
| 500 | 200 | 400 | | 25 | 10 | 20 |
| 250 | 100 | 200 | | 12.5 | 5 | 10 |
| 100 | 40 | 80 | | 5 | 2 | 4 |
| 25 | 10 | 20 | | 1.25 | 0.5 | 1 |
| 12.5 | 5 | 10 | | 0.625 | 0.25 | 0.5 |
| 5 | 2 | 4 | | 0.25 | 0.1 | 0.2 |
| 2.5 | 1 | 2 | | 0.125 | 0.05 | 0.1 |
| 1000 | 1000 | 200 | Acidified plasma | 50 | 50 | 10 |
| 500 | 500 | 100 | | 25 | 25 | 5 |
| 200 | 200 | 40 | | 10 | 10 | 2 |
| 40 | 40 | 8 | | 2 | 2 | 0.4 |
| 10 | 10 | 2 | | 0.5 | 0.5 | 0.1 |
| 2 | 2 | 0.4 | | 0.1 | 0.1 | 0.02 |
| 1 | 1 | 0.2 | | 0.05 | 0.05 | 0.01 |

these compounds in methanol to yield the target concentrations. AcMPAG stock solutions (1 mg/mL and 5 mg/mL) were prepared in HPLC grade methanol acidified with 0.3 g/L phosphoric acid. All stock solutions were stored at −80 °C.

MPA, MPAG and AcMPAG working solutions were prepared by diluting the stock solutions in the appropriate volume of HPLC grade methanol acidified with 0.3 g/L phosphoric acid to yield the concentrations listed in Table 1. The internal standard working solutions (300 and 900 ng/mL) were prepared from the 1 mg/mL stock solutions at the time of assay by dilution in methanol and kept at 4 °C.

Calibration standards were prepared by diluting 25 μL of MPA, MPAG and AcMPAG working solutions with 0.475 mL matrix (plasma, urine or NaCl) to yield the calibration standard concentrations described in Table 1. Human plasma and urine were acidified with 8 and 4 μL, respectively, of 85% phosphoric acid per 0.5 mL of matrix, and samples used to measure free MPA were prepared in a 9 g/L sodium chloride solution at pH 7.4, as previously validated [25]. Blank standards for the calibration curves consisted of 0.100 μL of drug-free matrix (acidified plasma, urine or NaCl). Quality control (QC) samples (at low, medium and high concentrations) were prepared in glass tubes by diluting stock solutions in matrix; urine and plasma samples were acidified, whereas samples for free MPA analysis were prepared in NaCl solution. To assess the stability of free MPA (see Section 2.6), QC samples used during validation were also prepared in non-acidified plasma. QC sample concentrations are noted in Table 2. Calibration standards and QC aliquots were stored at −80 °C.

### 2.3.2. Plasma and urine sample extraction

Solid-phase extraction of MPA, MPAG and AcMPAG from acidified urine and plasma samples was processed using modifications to previously published methods [26,27]. The choice of the internal standard (indomethacin) was also based on other published reports [22,28]. Specific modifications are as follows. All samples were thawed at room temperature before use. Urine samples were centrifuged at 300 × *g* for 5 min at 4 °C prior to use, whereas non-acidified plasma samples (500 μL) used to measure concentrations of free (or unbound) MPA were applied to Centrifree tubes (Millipore Corporation, Bedford, MA) and centrifuged at 4500 × *g* for 20 min at 20 °C. The internal standard (100 μL of indomethacin at 900 ng/mL, or at 300 ng/mL for free MPA determination) and 1 mL of 0.1 M HCl were added to standard, quality control and patient samples (100 μL) in glass tubes. Samples were vortex-mixed for 30 s and applied to Strata-X 60 mg columns (Phenomenex, Torrance, CA) that had been conditioned with 1 mL methanol followed by 2 mL of 0.1 M HCl. The loaded cartridges were then sequentially washed with 2 mL of 0.1 M HCl and 2 mL of 25% methanol. The cartridges were dried under full vacuum. Afterwards, the analytes were eluted with 2 mL methanol. Prior to analysis, methanol was evaporated to dryness under nitrogen at 20 °C for 30 min with a Turbo Vap system (Zymark Corporation, Hopkinton, MA). The residue was diluted in 100 μL (for plasma) and 500 μL (for urine) of 50% methanol containing 3 mM ammonium formate and 0.5% acetic acid.

Table 2
Assay imprecision

| | | | MPA | | | MPAG | | | AcMPAG | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | QC LOW | QC MED | QC HIGH | QC LOW | QC MED | QC HIGH | QC LOW | QC MED | QC HIGH |
| Plasma | QC concentrations (mg/L) | | 0.15 | 25.00 | 40.00 | 0.15 | 25.00 | 40.00 | 0.03 | 5.00 | 8.00 |
| | Intra-day | Average (mg/L) | 0.142 ± 0.007 | 21.67 ± 0.31 | 35.98 ± 0.50 | 0.133 ± 0.007 | 23.62 ± 0.94 | 40.71 ± 0.92 | 0.029 ± 0.003 | 4.66 ± 0.04 | 8.51 ± 0.09 |
| | | CV (%) | 4.57 | 1.43 | 1.38 | 4.93 | 3.97 | 2.26 | 9.12 | 0.78 | 1.02 |
| | | Bias (%) | −5.11 | −13.32 | −10.05 | −11.33 | −5.54 | 1.79 | −3.33 | −6.81 | 6.43 |
| | Inter-day | Average (mg/L) | 0.141 ± 0.003 | 21.01 ± 0.57 | 34.67 ± 1.26 | 0.14 ± 0.01 | 22.35 ± 1.34 | 38.41 ± 2.25 | 0.028 ± 0.002 | 4.42 ± 0.21 | 8.01 ± 0.46 |
| | | CV (%) | 4.79 | 3.01 | 3.50 | 8.72 | 5.83 | 5.71 | 9.49 | 4.61 | 5.55 |
| | | Bias (%) | −5.93 | −15.95 | −13.33 | −6.37 | −10.62 | −3.98 | −7.41 | −11.61 | 0.07 |
| NaCl | QC concentrations (mg/L) | | 0.03 | 5.00 | 8.00 | – | – | – | – | – | – |
| | Intra-day | Average (mg/L) | 0.03 ± 0.002 | 5.07 ± 0.24 | 7.76 ± 0.10 | – | – | – | – | – | – |
| | | CV (%) | 7.61 | 4.74 | 1.32 | – | – | – | – | – | – |
| | | Bias (%) | 1.11 | 1.45 | −3.00 | – | – | – | – | – | – |
| | Inter-day | Average (mg/L) | 0.03 ± 0.003 | 4.97 ± 0.12 | 8.19 ± 0.54 | – | – | – | – | – | – |
| | | CV (%) | 9.17 | 4.09 | 6.25 | – | – | – | – | – | – |
| | | Bias (%) | 4.07 | −0.66 | 2.39 | – | – | – | – | – | – |
| Urine | QC concentrations (mg/L) | | 0.38 | 25.00 | 40.00 | 0.15 | 10.00 | 16.00 | 1.50 | 20.00 | 32.00 |
| | Intra-day | Average (mg/L) | 0.41 ± 0.05 | 24.93 ± 0.36 | 38.9 ± 1.51 | 0.16 ± 0.02 | 10.3 ± 0.34 | 17.62 ± 0.85 | 1.41 ± 0.03 | 20.7 ± 0.80 | 32.2 ± 1.52 |
| | | CV (%) | 11.13 | 1.44 | 3.88 | 10.18 | 3.34 | 4.84 | 1.78 | 3.88 | 4.71 |
| | | Bias (%) | 9.16 | −0.28 | −2.82 | 5.33 | 3.19 | 10.15 | −6.02 | 3.49 | 0.67 |
| | Inter-day | Average (mg/L) | 0.42 ± 0.03 | 25.1 ± 0.71 | 39.4 ± 0.15 | 0.160 ± 0.008 | 10.5 ± 0.25 | 17.6 ± 0.16 | 1.427 ± 0.005 | 20.4 ± 0.55 | 31.5 ± 0.35 |
| | | CV (%) | 10.22 | 3.56 | 3.51 | 8.17 | 4.31 | 3.44 | 6.85 | 4.23 | 3.91 |
| | | Bias (%) | 11.44 | 0.32 | −1.40 | 6.96 | 5.40 | 10.15 | −4.90 | 2.23 | −1.46 |

### 2.4. Analytical system and conditions

HPLC was performed at ambient temperature on an Alliance 2690 HPLC system (Waters, Milford, MA). Separation was performed on a Gemini $C_{18}$ column (100 mm × 4.6 mm, particle size 5 μm) from Phenomenex (Torrance, CA). A binary mobile phase, consisting of $H_20$ with 3 mM ammonium formate and 0.5% acetic acid (solvent A), and methanol with 3 mM ammonium formate and 0.5% acetic acid (solvent B) was used at a flow rate of 0.9 mL/min. The analytes were eluted using the following program: 0–1 min, isocratic 65% B; 1–3 min, linear gradient 65–85% B; 3–6 min, isocratic 85% B; 6–6.1 min, linear gradient 85–65% B; 6.1–9 min 65% B.

Mass spectrometry was performed with an API-3000 triple quadrupole mass spectrometer (Applied Biosystems-Sciex, Concord, ON, Canada) equipped with a turbo ion-spray source. Electrospray ionization was performed in the positive-ion mode with an ionization voltage of 5000 V, an orifice voltage of 100 V, collision energy of 25 V, and a heater probe temperature of 500 °C. MPA, MPAG, AcMPAG and indomethacin were detected using the following mass transitions: 321.1 → 207.2 (MPA), 514.3 → 321.2 (MPAG and AcMPAG) and 358.2 → 139.1 (indomethacin). Analytes were quantified using integrated peak area ratios of MPA/indomethacin, MPAG/indomethacin or AcMPAG/indomethacin by MassChrom 1.1.2 software (Applied Biosystems-Sciex, Concord, ON, Canada).

### 2.5. Pharmacokinetic analysis

For each patient, the pharmacokinetics of MPA, MPAG and AcMPAG in plasma were analyzed using noncompartmental methods by WinNonLin v5.01 software (Pharsight, Mountain View, CA); the area under the concentration–time curve from 0 to 6 h ($AUC_{0–6}$), $AUC_{6–12}$ and $AUC_{0–12}$ were calculated using the linear trapezoidal method in WinNonLin. As described [12], an $AUC_{0-\infty}$ could not be estimated because a secondary peak of variable size in samples from several individuals prohibited the estimation of the slope. $C_{max}$ was the highest observed plasma concentration, and $T_{max}$ was the time of $C_{max}$.

### 2.6. Method validation

The method described above, developed to quantify free MPA, total MPA, MPAG and AcMPAG in human plasma and urine, was validated by analysis of quality control samples. The intra- and inter-day precision (coefficient of variation, CV (%)) and accuracy (bias (%), calculated as: (measured QC concentration − reference QC concentration)/reference QC concentration) were determined by analyzing three replicates of QC samples on three different days.

A seven-point calibration curve was prepared by spiking blank plasma, sodium chloride solution or urine with appropriate amounts of each analyte with concentrations up to 50 mg/L for MPA and MPAG and 40 mg/L for AcMPAG (Table 1). The linear regression of MPA, MPAG and AcMPAG/indomethacin peak area ratios was weighted by $1/x^2$. The coefficient of determination ($R^2$) 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.

The recovery after the extraction procedure was determined by comparing the peak areas of QC samples prior to and after extraction. Results are expressed as a percentage area of the extracted QC relative to the directly injected reference standard.

Stability of the analytes in whole blood and in plasma at 4 °C was investigated to evaluate how long patients' samples would be stable on ice before (in whole blood) and after centrifuga-

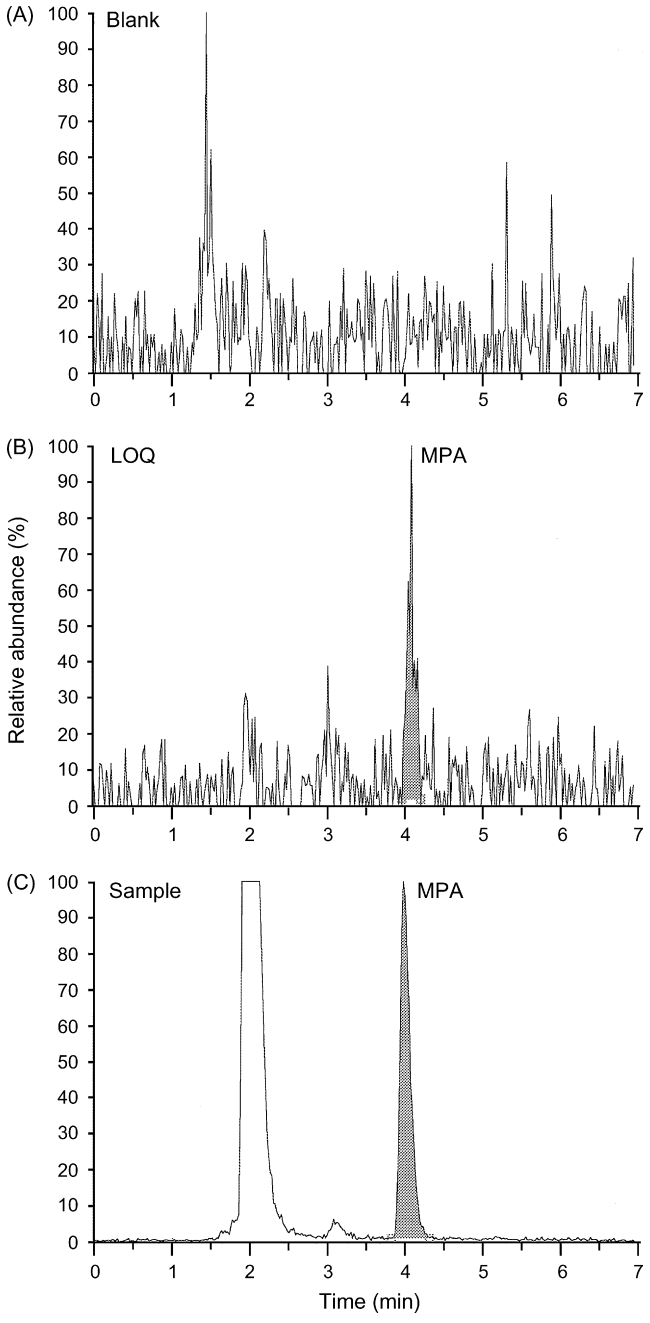


Fig. 1. Chromatograms of free MPA in non-acidified plasma; a blank (A), the LLOQ (B), and a patient sample (C) are presented. Free MPA concentration in this sample was 0.125 mg/L.

tion (in plasma). Stability in blood was assessed by keeping the samples in an ice bath for up to 75 min, and in plasma for up to 25 min. Short-term (benchtop) and long-term stability of the analytes in plasma was investigated at QC low and high concentrations by analyzing samples stored at 20 °C for 4 h and at −80 °C for 185 days. The same procedure was used to study the stability in urine. 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 (30 min) for three cycles. Stability of the processed samples in the autosampler 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 and internal standard after storage at −80 °C for 185 days. The effect of dilution of MPAG samples over the quantification range was also assessed. All stability evaluations were performed in triplicate.

## 3. Results and discussion

Representative chromatograms in non-acidified and acidified plasma and in urine are presented in Figs. 1–3, respectively. The retention times of MPA, MPAG, AcMPAG and an internal standard (indomethacin) were 4.05, 1.90, 2.94 and 5.65 min, respectively.

For plasma samples, the validated assay was linear in the range of 0.05 to 50 mg/L for total MPA (slope: 0.053, $R^2$: 0.996) and MPAG (slope: 0.129, $R^2$: 0.997), from 0.01 to 10 mg/L for AcMPAG (slope: 0.185, $R^2$: 0.997), and from 0.01 to 10 mg/L for free MPA (slope: 0.004, $R^2$: 0.999). For urine samples, linearity extended from 0.125 to 50 mg/L for MPA (slope: 0.038, $R^2$: 0.996), 0.05 to 20 mg/L for MPAG (slope: 0.077, $R^2$: 0.992), and 0.1 to 40 mg/L for AcMPAG (slope: 0.111, $R^2$: 0.995). Samples yielding a concentration above the linear range were diluted and reanalyzed.

Table 2 lists the inter- and intra-day precision (CV (%)) and accuracy (bias (%)) for the measurement of analyte concentrations. For plasma samples, the extraction efficiency was 82–92% for MPA, 80–92% for MPAG, and 71–92% for AcMPAG. For urine samples, the extraction efficiency was 86.7–92.3% for MPA, 88.6–92.6% for MPAG, and 82.3–90.6% for AcMPAG.

For plasma samples, the LLOQ was 0.05 mg/L for MPA and MPAG, 0.01 mg/L for free MPA, and 0.01 mg/L for AcMPAG. For urine samples, LLOQ was 0.125 for MPA, 0.05 for MPAG, and 0.1 for AcMPAG.

Table 3 presents the stability of the analytes in whole blood and in plasma at 4 °C; results are expressed as the bias (%) when compared to $T = 0$. The bias (%) for the stability of the analytes in urine was −0.4% for MPA and −0.5% for MPAG after 16 h, and −18.2% for AcMPAG after 8 h. AcMPAG was much less

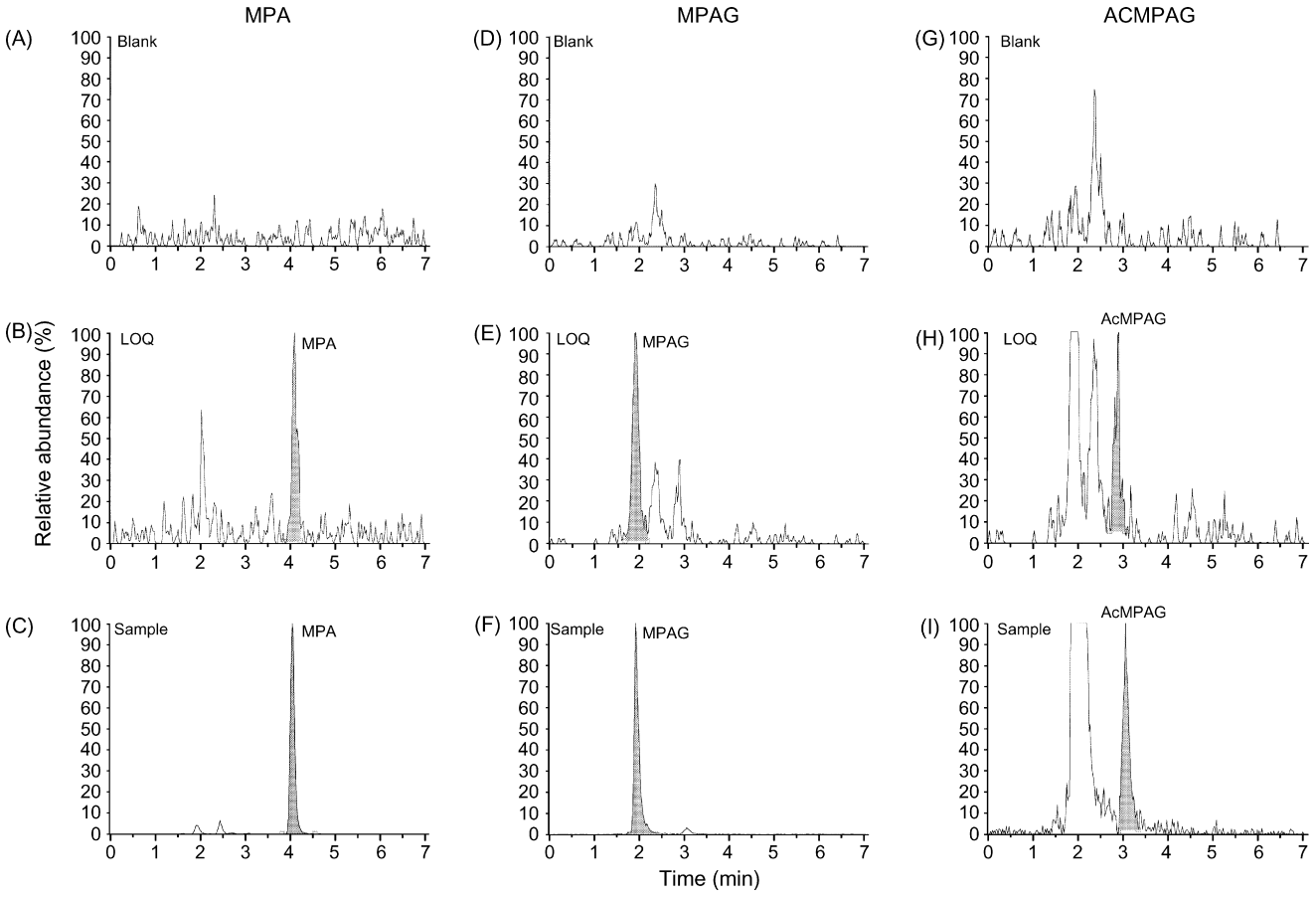


Fig. 2. Chromatograms of MPA (A–C) and its metabolites MPAG (D–F) and AcMPAG (G–I) in acidified plasma. For each analyte, a blank, the LLOQ, and a patient sample are presented. MPA, MPAG and AcMPAG concentrations in this sample were 17.03, 3.184 and 0.122 mg/L, respectively.

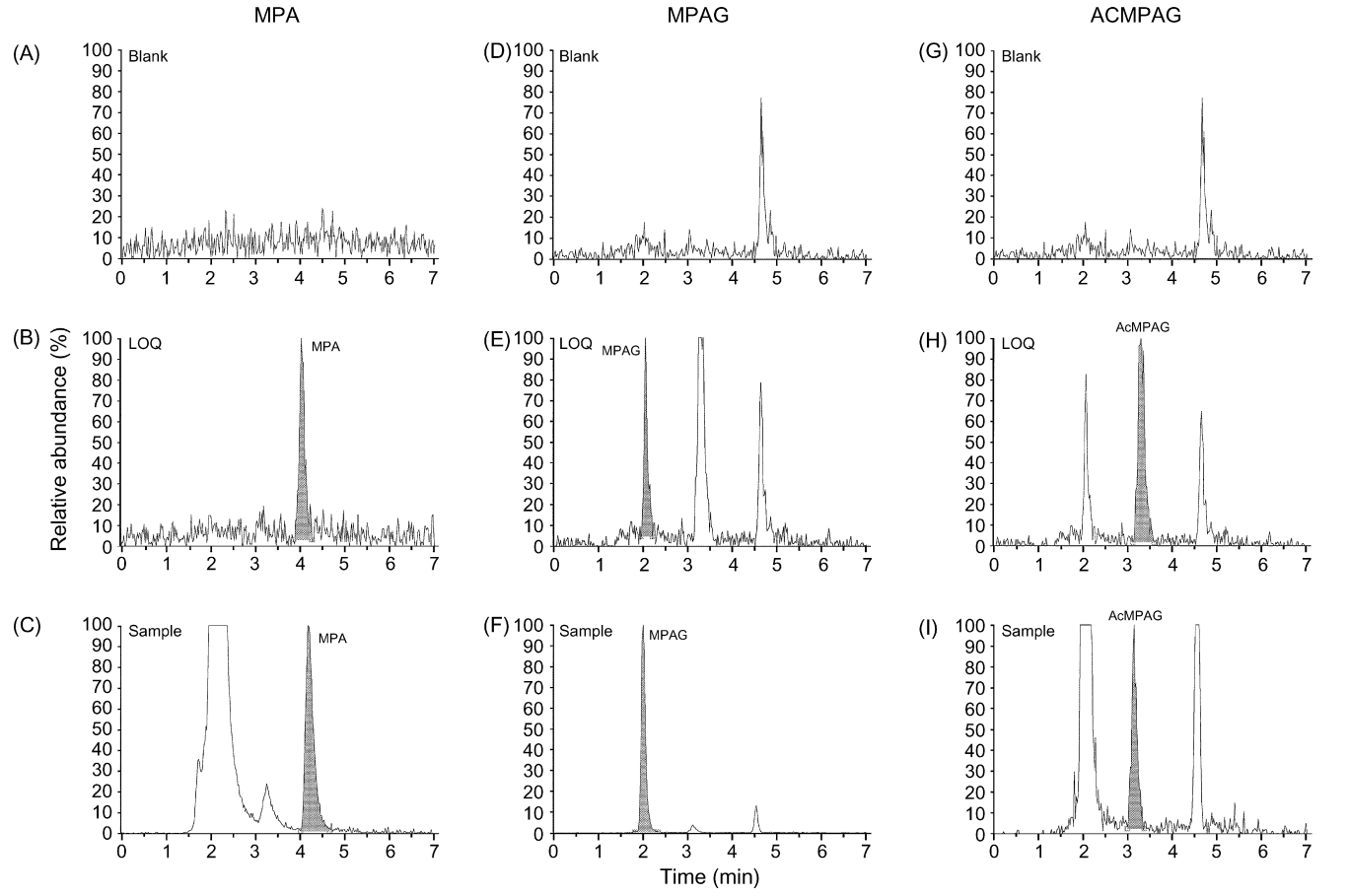


Fig. 3. Chromatograms of MPA (A–C) and its metabolites MPAG (D–F) and AcMPAG (G–I) in urine. For each analyte, a blank, the LLOQ, and a patient sample are presented. MPA, MPAG and AcMPAG concentrations in this sample were 24.801, 2275.0 and 49.70 mg/L, respectively.

stable than MPA or MPAG at 4 °C, and thus samples should be frozen rapidly after collection to obtain reliable results. Table 4 presents the results for the short-term (4 h at 20 °C) and long-term (185 days at −80 °C) stability, freeze/thaw stability, and stability of the processed samples in the autosampler (at 4 °C); values are expressed as the CV (%). Less than 5% of the nominal values of the stock solutions were lost over a 6-month period. The dilution of a 40 mg/L MPAG solution by a factor of 100 in six replicates was associated with a CV of 2.1% in plasma and 7.0% in urine.

Table 3
Stability of the analytes in blood and plasma at 4 °C

| Time in blood (min) | Time in plasma (min) | % bias vs. $T=0$ | | |
|---|---|---|---|---|
| | | MPA | MPAG | AcMPAG |
| 0 | 0 | – | – | – |
| | 25 | 10.1 | 2.3 | −2.8 |
| 15 | 0 | 10.7 | 1.0 | −0.8 |
| | 25 | 5.1 | −4.7 | −5.3 |
| 30 | 0 | 10.7 | 1.4 | −1.3 |
| | 25 | 9.3 | −0.7 | −5.8 |
| 45 | 0 | 8.6 | 0.0 | −7.3 |
| | 25 | 7.0 | −1.2 | −4.9 |
| 60 | 0 | 12.9 | 2.4 | −3.2 |
| | 25 | 11.2 | 2.0 | −4.1 |
| 75 | 0 | 11.8 | 1.3 | −3.3 |
| | 25 | 1.1 | −6.7 | −13.7 |

Amongst several quantification techniques that have been used to measure MPA in plasma, the EMIT immunoassay [20] is simple and requires minimal sample pre-treatment. However, its main weaknesses are that it does not measure MPA glucuronide metabolites and it is less specific than other methods. Indeed, cross-reactivity with AcMPAG leads to a methodical positive bias when results are compared with those from HPLC [29–31]. Moreover, methods using HPLC followed by UV detection [16–19] enable specific measurement of MPA and its metabolites. However, these methods entail longer assay times (14–25 min), interferences can lead to problems in AcMPAG quantitation, and quantification limits are often higher than methods using HPLC–MS/MS. On the other hand, very few studies have validated the use of HPLC–MS/MS for the purpose of quantifying MPA and its metabolites [21–23]. Indeed, only one such method has been validated for each of AcMPAG [21] and free MPA [23] quantification. Moreover, no methods have been validated that simultaneously measure total MPA, free MPA, MPAG and AcMPAG, and that are compatible with different matrices (plasma and urine). The method we describe herein has been validated for all these aspects.

Table 4
Stability of the analytes

| | | MPA (mg/L) | | MPAG (mg/L) | | AcMPAG (mg/L) | |
|---|---|---|---|---|---|---|---|
| | | Low | High | Low | High | Low | High |
| Acidified plasma | | | | | | | |
| Concentrations (mg/L) | | 0.15 | 40.0 | 0.15 | 40.0 | 0.03 | 8.0 |
| Short-term | Stability (bias (%)) | −3.1 | −13.3 | −9.8 | −7.3 | −12.2 | 3.3 |
| Long-term | | −5.3 | −17.1 | −10.2 | −9.9 | −11.1 | −1.2 |
| Freeze/thaw | | −6.2 | −13.8 | −13.3 | −7.1 | −1.1 | 3.7 |
| In the autosampler | | −5.3 | −17.5 | −14.2 | −12.6 | −8.9 | −7.1 |
| Non-acidified plasma | | | | | | | |
| Concentrations (mg/L) | | 0.15 | 40.0 | – | – | – | – |
| Short-term | Stability (bias (%)) | 1.6 | −5.9 | – | – | – | – |
| Long-term | | 3.3 | −6.4 | – | – | – | – |
| Freeze/thaw | | −1.1 | −1.8 | – | – | – | – |
| In the autosampler | | 6.6 | −2.0 | – | – | – | – |
| NaCl | | | | | | | |
| Concentrations (mg/L) | | 0.03 | 8.0 | – | – | – | – |
| Short-term | Stability (bias (%)) | −7.8 | −10.5 | – | – | – | – |
| Long-term | | −7.8 | −5.1 | – | – | – | – |
| Freeze/thaw | | 2.2 | −4.8 | – | – | – | – |
| In the autosampler | | 0.6 | −0.7 | – | – | – | – |
| Urine | | | | | | | |
| Concentrations (mg/L) | | 0.375 | 40.0 | 0.15 | 16.0 | 1.5 | 32.0 |
| Short-term | Stability (bias (%)) | −1.7 | −9.0 | 4.4 | 2.1 | −5.8 | −10.9 |
| Long-term | | −7.1 | −9.1 | −4.0 | 2.6 | −11.1 | −11.5 |
| Freeze/thaw | | 2.6 | −6.7 | −13.3 | 2.8 | −3.0 | −10.7 |
| In the autosampler | | 4.2 | −0.3 | 14 | 10.9 | 5.9 | −2.5 |

To demonstrate the applicability of the method, serial plasma and urine samples were obtained from healthy volunteers enrolled in a pharmacogenetic study of MPA pharmacokinetics [24]. The samples were collected, processed and analyzed as described in this method. Fig. 4 shows representative curves for plasma concentration over time for free MPA, total MPA, MPAG and AcMPAG. The secondary peak at ∼475 min in these curves is typical of MPA pharmacokinetics and is due to enterohepatic (re)cycling of the drug [6]. Table 5 presents the results of the pharmacokinetic analysis. Previous studies

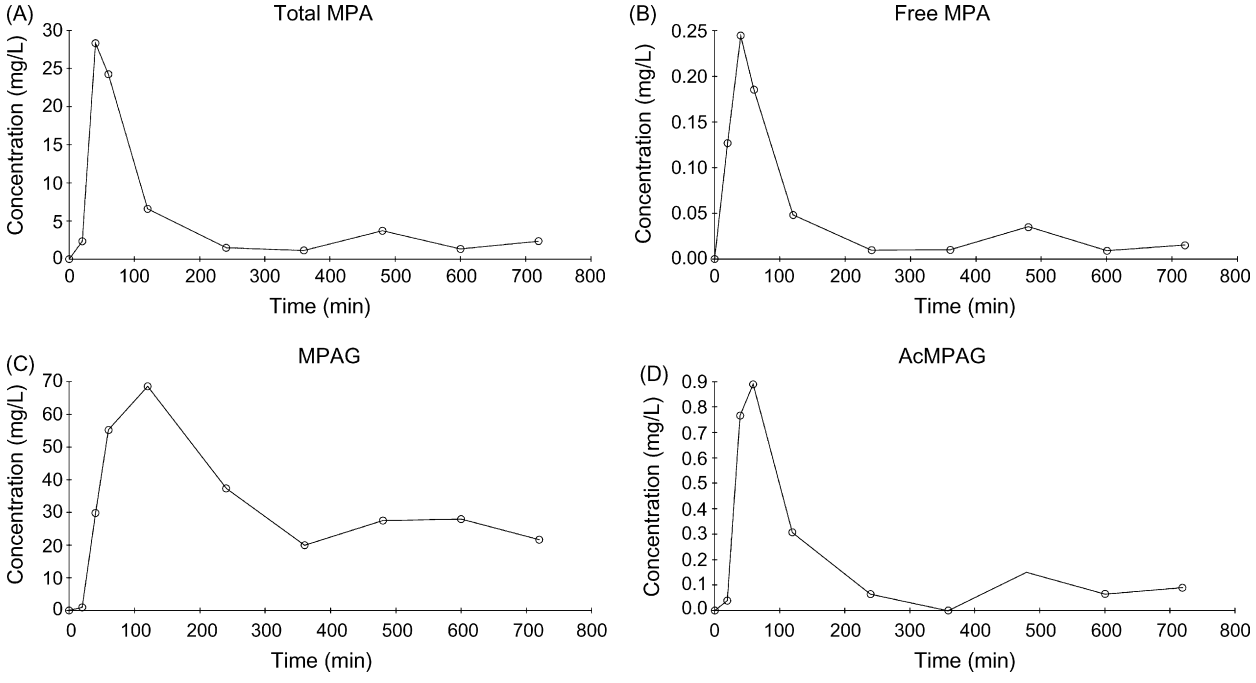


Fig. 4. Concentration–time profiles for total MPA (A), free MPA (B), MPAG (C) and AcMPAG (D) from an adult volunteer after a single 1.5 g oral dose of MMF.

Table 5
Pharmacokinetic parameters of 52 healthy volunteers dosed with MMF

| | Value (mean ± S.D.) | Variability (%) |
|---|---|---|
| Number of subjects | 52 | |
| $T_{max}$ (h) | | |
| Total MPA | 0.86 ± 0.35 | 40 |
| Unbound MPA | 0.88 ± 0.42 | 47 |
| Total MPAG | 1.83 ± 0.38 | 21 |
| Total AcMPAG | 1.03 ± 0.42 | 41 |
| $C_{max}$ (mg/L) | | |
| Total MPA | 25.57 ± 8.15 | 32 |
| Unbound MPA | 0.25 ± 0.18 | 71 |
| Total MPAG | 48.85 ± 16.80 | 34 |
| Total AcMPAG | 0.93 ± 0.50 | 54 |
| $AUC_{0–12}$ (mg h/L) | | |
| Total MPA | 60.70 ± 15.65 | 26 |
| Unbound MPA | 0.52 ± 0.17 | 33 |
| Total MPAG | 312.34 ± 92.57 | 30 |
| Total AcMPAG | 2.65 ± 1.43 | 54 |
| Free fraction | | |
| MPA free fraction (%) | 0.85 ± 0.15 | 17 |
| EHC ($AUC_{6–12}/AUC_{0–12}$) | | |
| Total MPA | 24.66 ± 5.91 | 24 |
| Free MPA | 23.95 ± 8.17 | 34 |
| MPAG | 39.05 ± 6.08 | 16 |
| AcMPAG | 24.02 ± 6.45 | 27 |
| Urinary excretion 0–12 h (mg) | | |
| MPA | 3.84 ± 3.69 | 96 |
| MPAG | 766.06 ± 218.65 | 29 |
| AcMPAG | 8.33 ± 3.91 | 47 |

of both healthy volunteers and transplant patients have demonstrated that the pharmacokinetics of MPA and its metabolites are highly variable [6,12,32–36], and our current results support these findings (Table 5). About 97% of MPA is bound to plasma proteins, especially albumin, but only free MPA is pharmacologically active, justifying its evaluation in pharmacokinetic studies [4]. A relationship between outcome and exposure to total MPA [12,13,37–39], free MPA [12,32,40] or AcMPAG [41] has been shown in several transplant populations, justifying the need to develop precise tools to measure MPA and its metabolites. Although AcMPAG is active [7,9] and potentially toxic [8,41,42], it is rarely measured in pharmacokinetic studies, possibly because very few methods have been developed to reliably quantify this metabolite.

## 4. Conclusion

Our new HPLC–MS/MS method has excellent analytical sensitivity and specificity, and moreover, it allows simultaneous monitoring/quantification of total and free MPA as well as its metabolites in both plasma and urine. Having a lower LLOQ for MPA and its metabolites, this method further permits quantification of lower concentrations than most HPLC/UV methods [16,19,26,43–45]. For the first time, MPA and both of its metabolites have been simultaneously assessed, and our findings are consistent with the only report of the pharmacokinetics of these analytes in healthy volunteers and confirm the previously suggested wide interindividual variability of metabolite levels [4].

Advantages of this method include a wide concentration range of detection, the small sample volume required for the assay (100 μL), and a relatively short assay time (6 min), allowing moderate throughput. This method is suitable for large-scale applications in pharmacokinetic studies of MPA and its metabolites, and thus can facilitate the therapeutic drug monitoring of MPA and optimize MMF efficacy in transplant recipients.

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