

High-Performance Liquid Chromatography Method for the Determination of Mycophenolic Acid and Its Acyl and Phenol Glucuronide Metabolites in Human Plasma

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Abstract: Measuring the concentration of the pharmacologically active metabolite of mycophenolic acid (MPA), acyl-MPAG (AcMPAG), in addition to the pharmacologically inactive phenol glucuronide metabolite (MPAG) may prove useful in the therapeutic drug monitoring of MPA. A simple high-performance liquid chromatography method with ultraviolet detection (HPLC-UV) was established for simultaneous determination of MPA, AcMPAG, and MPAG in human plasma. The method utilizes 2 internal standards (IS), phenolphthalein glucuronic acid (PGA) for MPAG and a carboxy butoxy derivative of MPA (MPAC) for AcMPAG and MPA. The method consists of solid-phase extraction of the analytes followed by analysis over a Zorbax Rx C₈ column (150 × 4.6 mm, 5 μm) at 254 nm. The analytes were separated with a gradient mixture of methanol and 0.1% phosphoric acid over a run time of 14 minutes at a flow rate of 1 mL/min. The assay was linear in the concentration range from 0.2 to 50 mg/L for MPA, 0.5 to 25 mg/L for AcMPAG, and 2 to 500 mg/L for MPAG. The mean ± SD interday accuracy and %CV for MPA were 100.3 ± 5.7 and 5.7%, for AcMPAG, 102.6 ± 5.7 and 5.6%, and for MPAG 100.5 ± 5.3 and 5.3%, respectively. The average ± SD of MPA, MPAG, and AcMPAG maximum concentrations (C_{max}) in 23 kidney transplant recipients on 500 or 1000 mg twice daily mycophenolate mofetil were 11.77 ± 9.43, 88.15 ± 46.4, and 3.01 ± 1.73 mg/L, respectively, and the predose trough (C_{min} morning) concentrations were 2.24 ± 3.11, 55.44 ± 29.55, and 1.42 ± 0.74 mg/L, respectively. The method described is robust, sensitive, reproducible, and will be useful in therapeutic drug monitoring or pharmacokinetic studies of MPA.

Key Words: MPA, MPAG, AcMPAG, HPLC-UV, assay, concentration
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Mycophenolic acid is an immunosuppressive agent commonly used in solid organ transplantation. It is administered either as the morpholinoethylester prodrug mycophenolate mofetil (MMF, Cellcept[®], Roche Pharmaceuticals)¹ or as a sodium salt, mycophenolate sodium (Myfortic[®], Novartis

Pharmaceuticals).² It undergoes metabolism primarily by the uridine diphosphate glucuronosyl transferase (UDP-GT) system at liver, kidney, and intestine.^{3–5} The major metabolite of MPA is the pharmacologically inactive phenol glucuronide metabolite MPAG (also known as 7-O-glucuronide metabolite). A second, less studied but important acyl glucuronide metabolite (AcMPAG) has been identified and is believed to have similar pharmacological and/or toxicological actions to MPA^{6,7}; however, the concentration–time profile of this metabolite in relation to clinically manifested adverse reactions has not been well characterized in different groups of transplant recipients.

Because of lack of commercially available immunoassay methods for determination of MPA in addition to its phenyl and acyl metabolites, liquid chromatography–based methods remain essential for determination of MPA in human plasma. A number of HPLC-UV methods have been published on the assay development for quantification of total MPA and MPAG in human plasma,^{8–13} but to date only 2 have been described to quantify AcMPAG in addition to MPA and MPAG in a single chromatographic run.^{14,15} Previously we have reported a simple and reproducible HPLC-UV method for determination of MPA and MPAG.¹⁶ We now have modified this method to quantify AcMPAG and have improved the limit of quantification for MPA and MPAG analysis. In addition, the modified method has been revalidated thoroughly according to the Guidelines for Bioanalytical Method Validation published by the Food and Drug Administration (FDA) of the United States.¹⁷

MATERIALS AND METHODS

Plasma Samples

Drug-free plasma samples from healthy donors (n = 6) were obtained from Rhode Island Blood Center (Providence, RI), pooled, and used to prepare the in-house calibration standards and QCs. In addition, as part of a clinical pharmacokinetic study approved by the Institutional Review Board at Rhode Island Hospital, serial blood samples were collected from 23 kidney transplant recipients over a period of 12 hours after MMF administration in ethylenediaminetetraacetic acid anticoagulant tubes. The blood samples were centrifuged at 15,000 × g for 10 minutes, and the plasma was stored at –80°C until analysis. All patients were on 500 or 1000 mg twice a day dose of mycophenolate mofetil.

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Chemicals

Standard samples of MPA, AcMPAG, MPAG, and internal standard (IS) for MPA and AcMPAG, a carboxy butoxy ether derivative of MPA (MPAC), were kindly donated by Roche Pharmaceuticals (Palo Alto, CA). The sample of MPAG was originally synthesized by the Analytical Services International Ltd, London, UK and was 98.7% pure with less than 0.1% MPA impurity. AcMPAG standard was 97.75% pure. Phenolphthalein glucuronic acid (PGA) used as IS for MPAG determination was purchased from Sigma (St. Louis, MO).

Before use, all glass and plastic ware was treated with AquaSil™ Siliconizing Fluid (Pierce, Rockford, IL). All solvents were HPLC grade, and all reagents were analytic grade. HPLC quality deionized water was prepared using Milli Q50 (Millipore, Bedford, MA) water purification system. HPLC grade methanol was purchased from Pharmco Products Inc (Brookfield, CT), and phosphoric acid (85% vol/vol) ACS reagent was purchased from Sigma-Aldrich (St. Louis, MO).

Calibration Curves

Stock solutions containing 500 mg/L MPA, 1000 mg/L AcMPAG, and 2500 mg/L MPAG were prepared in absolute methanol. Stock solutions of MPA and MPAG were stored at -20°C , whereas stock solution of AcMPAG was stored at -80°C until use. Aliquots of the stock MPA, AcMPAG, and MPAG solutions were diluted with drug-free plasma to give 6 combined calibration standards containing 0.2, 1.0, 5.0, 10.0, 25.0, and 50.0 mg/L MPA, 0.5, 2.5, 5.0, 10.0, 15.0, and 25.0 mg/L AcMPAG, and 2.0, 10.0, 25.0, 150.0, 250.0, and 500.0 mg/L MPAG. Three in-house quality control standards (QCs), representing the low, medium, and high concentrations, were prepared in drug-free plasma with a final concentration of 0.5, 7.5, and 30.0 mg/L MPA, 0.75, 7.5, and 20 mg/L AcMPAG, and 5.0, 100.0, and 300.0 mg/L MPAG. In addition, 3 reference standards containing the same concentrations of MPA, AcMPAG, and MPAG as the QCs were prepared in methanol-water (50:50% vol/vol). The working solution of IS contained 20 mg/L MPAC and 200 mg/L PGA. All calibration, quality control, and reference standards and the combined IS solutions were aliquoted and stored at -20°C until use.

Sample Extraction

Calibrators, QCs, or patient plasma samples were thawed at 37°C using a reciprocal shaking water bath. Mycophenolic acid, AcMPAG, and MPAG were extracted from plasma matrix using Isolute C₂, 100 mg, 3 mL SPE cartridges (Argonaut Technologies Inc. Foster City, CA). To 100 μL of the samples, 100 μL of combined internal standard solution and 1 mL of 1% vol/vol phosphoric acid (pH = 1.5) were added, and samples were vortex mixed. This was then loaded onto SPE cartridges mounted on a VisiPrep®DL SPE manifold (Supelco, Bellefonte, PA) previously primed with 2 mL of methanol and 2 mL of 0.1% vol/vol phosphoric acid (pH = 2.18) and allowed to drain. The cartridges were then washed in 2 steps. The first step consisted of washing with 2 mL of 0.1% vol/vol phosphoric acid solution, and, after complete drainage, with another 1 mL of 0.1% vol/vol phosphoric acid solution. After complete drainage the cartridges were subjected to 10 minutes of full vacuum. The analytes were then eluted with 300 μL of methanol-

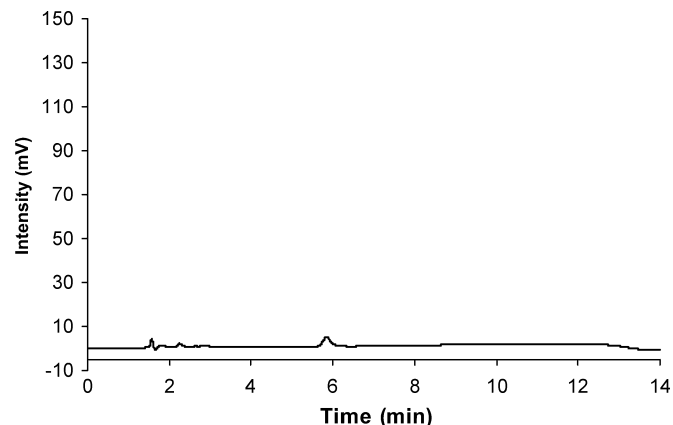


FIGURE 1. Chromatogram depicting blank extracted plasma.

0.1% phosphoric acid (70:30% vol/vol, pH = 3.26), and 50 μL of this was injected at room temperature onto the analytic column.

HPLC Apparatus and Conditions

The chromatographic separation was performed on a Hitachi D-7000 series instrument (San Jose, CA) consisting of an autosampler fitted with a 200- μL sample loop, a quaternary pump, a column oven, and a variable-wavelength UV detector set at 254 nm. Peak areas were integrated using the Hitachi System Manager (HSM) software. Mobile phase was filtered and degassed using 0.45 μm Nylon filters (Millipore, Bedford, MA) under vacuum. Chromatographic separation of individual analytes was achieved using a Zorbax Rx C₈, 150 \times 4.6 mm, 5 μm particle size (Agilent Technologies, Palo Alto, CA) analytic column preceded by a Supelco 1/16", peek, 2 μm frit, precolumn filter (Supelco, Bellefonte, PA) maintained at 35°C .

The flow rate remained at 1 mL/min throughout the 14-minute run. For the first 2.2 minutes of each run, the mobile phase remained at methanol-0.1% vol/vol phosphoric acid (48:52% vol/vol), from 2.2 to 7.0 minutes there was a continuous gradient change to methanol-0.1% vol/vol phosphoric acid (60:40% vol/vol), and from 7 to 10 minutes the

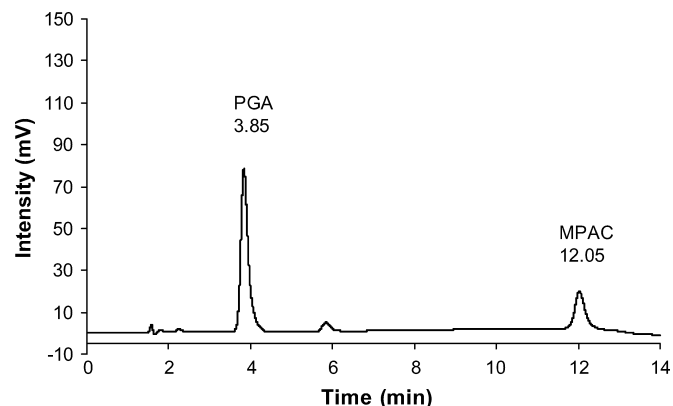


FIGURE 2. Chromatogram of drug-free plasma containing internal standards, 20 mg/L MPAC, and 200 mg/L PGA.

TABLE 1. Assay Parameters for Determination of Total MPA, AcMPAG, and MPAG

Component	Concentration Range (mg/L)	Retention Time (min)*	LOD (mg/L)	LLOQ (mg/L)	Regression Coefficient
MPA	0.2–50	10.85 ± 0.04	0.1	0.2	0.998–0.999
MPAC	N/A	12.05 ± 0.06	N/A	N/A	N/A
MPAG	2.0–500	4.58 ± 0.03	0.5	2.0	0.984–0.997
AcMPAG	0.5–25	7.56 ± 0.08	0.2	0.5	0.992–0.981
PGA	N/A	3.85 ± 0.02	N/A	N/A	N/A

*Mean ± SD of 10 replicates; LOD, limit of detection; LLOQ, lower limit of quantification; N/A, not applicable.

composition maintained at methanol-0.1% vol/vol phosphoric acid (60:40% vol/vol). After 10 minutes, the composition changed back to methanol-0.1% vol/vol phosphoric acid (48:52% vol/vol) with a fast gradient method (within 6 seconds). Calibration curves consisted of respective concentrations of MPA and AcMPAG plotted separately against their peak area ratios with MPAC as IS and MPAG concentration plotted against its peak area ratio with PGA as IS.

Method Validation

All assay validation steps were carried out according to the 2001 version of the FDA guidelines for the validation of analytic methods.¹⁷ The lower limit of quantification (LLOQ) was defined at a signal-to-noise ratio of 10:1, and limit of detection at signal-to-noise ratio of 3:1. The calibration curve was constructed by injecting triplicates of the extracted plasma-based calibrators inclusive of LLOQ and the quality controls on 10 different study days. The closeness of the validation results for QCs obtained by the method to the true value and the degree of agreement among the individual results for multiple analytic runs of the same sample were taken as the accuracy and precision, respectively.

Specificity was determined by comparing the retention times of each of the analytes against the chromatogram of extracted pooled plasma samples without drugs added

(10 replicates) and against extracted plasma samples that were spiked with only IS (n = 10). The peaks of each of the components were sought to be well resolved, and also there was no interference from endogenous or exogenous materials at the retention times of the analytes. The chromatograms were checked for consistency in retention times and concentration-dependent peak areas of the analytes.

The possibility of interference from other drugs with the retention times of the analytes of interest was evaluated by determining the chromatographic retention times of several commonly coadministered pharmacological agents. These agents included cyclosporine, tacrolimus, sirolimus, prednisone, prednisolone, ketoconazole, fluconazole, cimetidine, ranitidine, acetaminophen, aspirin, and theophylline. Blank plasma samples were spiked with a nominal concentration corresponding to the peak concentration of drug of interest at steady state¹⁸ and extracted with the SPE procedure described above, and 50 μ L of the eluent was injected into the HPLC column. To further evaluate interference, plasma samples from 21 kidney transplant patients who were not on MPA therapy were extracted and their chromatograms inspected for interfering peaks. These patients were on medications commonly administered to transplant recipients including calcineurin inhibitors (cyclosporine or tacrolimus), prednisone, lipid-lowering agents, and antihistamines.

Assay linearity was assessed using an unweighted linear regression method between the LLOQ (0.2, 0.5, 2.0 mg/L for MPA, AcMPAG, and MPAG, respectively) and the sample representing the upper limits (50.0, 25.0, 500.0 mg/L MPA, AcMPAG, MPAG, respectively) of clinically relevant concentrations in plasma.

The recovery of the extraction procedure was assessed by comparing the peak areas of the extracted QCs with peak areas of reference standards prepared in methanol-water (50:50% vol/vol) and injected directly onto the analytic column and is expressed as a percentage area of the extracted QC relative to the directly injected reference standard. The extraction procedure was modified so that the recovery of the extraction procedure remains consistent at the low, medium, and high QC concentrations.

Freeze-thaw, short-term, autosampler, and long-term stability studies were evaluated. To evaluate freeze-thaw stability, aliquots of the QC plasma samples were subjected to freezing for 24 hours at -20°C and thawed unassisted at room temperature for 3 cycles. Because the actual calibration and QC standards were thawed at 37°C , freeze-thaw stability was

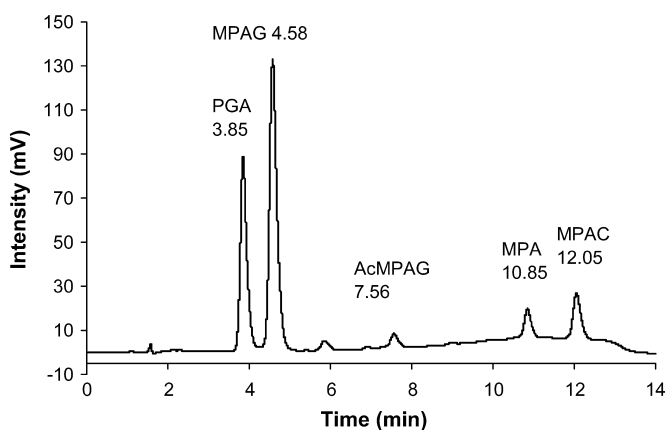


FIGURE 3. Chromatogram of a plasma sample from a representative kidney transplant recipient; the concentrations of MPA, AcMPAG, and MPAG were calculated to be 18.7, 6.97, and 168.37 mg/L, respectively.

TABLE 2. Assay Validation Results for Determination of Total MPA, AcMPAG, and MPAG

Sample	Nominal Concentration (mg/L)	Recovery* (%)	Interday Accuracy* (%)	Intraday Accuracy* (%)	Interday Precision (%)	Intraday Precision (%)
MPA						
LLOQ	0.2	N/A	99.9 ± 5.02	96.5 ± 6.0	5.0	4.2
QC1	0.5	93.0 ± 1.4	99.6 ± 7.6	102.2 ± 8.2	7.6	12.5
QC2	7.5	88.3 ± 3.6	101.2 ± 4.2	99.8 ± 5.2	4.1	3.0
QC3	30	86.1 ± 1.2	100.5 ± 6.1	101.0 ± 4.1	6.1	6.2
AcMPAG						
LLOQ	0.5	N/A	105.4 ± 6.2	108.6 ± 4.6	5.9	4.6
QC1	0.75	90.1 ± 1.9	103.6 ± 4.8	103.5 ± 3.1	4.6	3.2
QC2	7.5	88.3 ± 4.2	102.6 ± 4.1	106.0 ± 4.1	4.0	3.5
QC3	20	87.6 ± 4.7	98.9 ± 7.9	103.7 ± 6.7	8.0	7.0
MPAG						
LLOQ	2.0	N/A	94.8 ± 6.0	92.6 ± 5.9	6.4	3.2
QC1	5	95.5 ± 2.0	105.5 ± 5.6	108.3 ± 3.7	5.3	5.3
QC2	100	97.5 ± 5.4	101.9 ± 4.8	104.9 ± 1.5	4.7	3.1
QC3	300	92.2 ± 3.1	99.9 ± 5.0	101.0 ± 2.0	5.0	1.75

*Mean ± SD of 10 replicates; CV, coefficient of variation; QC, quality control.

also performed for 3 cycles of freeze and thaw consisting of freezing at -20°C and thawing at 37°C in the reciprocating water bath. Short-term benchtop stability was performed by placing samples on the benchtop for a period of 24 hours at room temperature, extracting and determining the concentration of each of the analytes, and comparing it with extracted samples that were freshly thawed. The samples were also studied for autosampler stability (postpreparative stability) by keeping the extracted QC samples unassisted in a non-temperature-controlled autosampler (room temperature: approximately 25°C) for 24 hours and then quantifying and comparing the concentration with freshly extracted QC samples. Long-term stability was evaluated by freezing QC samples for a period of 6 months, quantifying the analytes, and comparing the concentration with the original concentrations obtained during the initial validation stage. Stock solution stability was evaluated for stock solutions of analytes and IS by keeping aliquots of the stock at room temperature for

6 hours and comparing the peak area with that of freshly prepared stocks.

RESULTS

The mobile phase switches were necessary to facilitate elution of 3 analytes, MPA, AcMPAG, and MPAG, and 2 internal standards, MPAC and PGA, and to shorten the length of the chromatographic run. Several isocratic mobile phase compositions were initially tested but failed to elute all the analytes with acceptable resolution, especially between the peaks of AcMPAG and MPAG. Chromatograms of drug-free plasma extracted without the addition of internal standard (Fig. 1) and containing internal standards MPAC and PGA are shown (Fig. 2). Each of the 5 components was well resolved, and no interference was observed from plasma peaks at the elution times of these analytes.

TABLE 3. Stability Results for MPA, AcMPAG, and MPAG

Sample	Nominal Concentration (mg/L)	Freeze-Thaw Stability (%)	Short-Term Stability (%)	Long-Term Stability (%)	Autosampler Stability (%)
MPA					
QC1	0.5	97.1 ± 1.2	97.1 ± 1.2	99.5 ± 2.3	113.9 ± 8.3
QC2	7.5	97.3 ± 3.4	100.8 ± 5.3	103.8 ± 5.1	97.5 ± 5.3
QC3	30	99.7 ± 4.8	99.9 ± 3.8	103.1 ± 3.2	99.8 ± 4.2
AcMPAG					
QC1	0.75	90.1 ± 3.3	85.4 ± 9.8	100.9 ± 4.3	109.9 ± 8.8
QC2	7.5	94.3 ± 2.6	100.9 ± 4.8	102.8 ± 5.4	107.9 ± 4.4
QC3	20	91.2 ± 5.9	97.0 ± 5.8	103.6 ± 6.7	115.7 ± 3.2
MPAG					
QC1	5	103.8 ± 2.6	96.8 ± 3.2	101.8 ± 2.3	107.3 ± 4.3
QC2	100	104.3 ± 2.1	93.7 ± 2.1	98.3 ± 3.8	104.5 ± 2.8
QC3	300	102.6 ± 3.7	94.4 ± 2.8	96.5 ± 4.6	96.9 ± 6.6

The final extracts of blank plasma samples spiked with commonly used drugs did not show any peaks for cyclosporine, tacrolimus, sirolimus, theophylline, aspirin, acetaminophen, cimetidine, ranitidine, or fluconazole. Samples containing prednisone, prednisolone, and ketoconazole, however, had the retention times of 8.65, 9.30, and 8.64 minutes, respectively. These peaks did not interfere with the retention times of any of the analytes of interest whose retention times are given in Table 1. In addition, analysis of plasma samples from 21 transplant recipients receiving immunosuppressive agents other than MPA revealed no interfering peaks from possible endogenous or exogenous compounds.

The chromatographic separation of MPA, AcMPAG, and MPAG from a representative kidney transplant recipient on MMF therapy is illustrated in Figure 3. The assay parameters consisting of retention time, LOD, LLOQ of the analytes, and the regression coefficients are given in Table 1. Although FDA guidelines require inclusion of 6 calibration curves and QC samples, we have included the results of the assay validation consisting of recovery, accuracy, and precision data for 10 sets of calibration curves with QC samples (Table 2). The assay was linear in the concentration range from 0.2 to 50 mg/L for MPA with a mean ($n = 10$) regression equation of $[y = 0.0696x + 0.0169]$, 0.5 to 25 mg/L for AcMPAG $[y = 0.043x - 0.0547]$, and 2 to 500 mg/L for MPAG $[y = 0.01x + 0.0211]$. The recoveries of the 2 internal standards, PGA and MPAC, were found to be $87.0 \pm 4.1\%$ and $82.0 \pm 3.6\%$, respectively.

The results of the stability test that includes freeze-thaw, short-term, long-term, and autosampler or postpreparative stability have been given in Table 3. Although AcMPAG did show lower accuracy than MPA and MPAG after the freeze-thaw procedure, these values were still within the 15% limit according to standards acceptable by the FDA. Short-term benchtop stability test did not show significant changes in the concentration of any of the analytes when compared with samples obtained by extracting freshly thawed plasma controls. We did not observe any change in the concentration of analytes in the QCs and IS after extraction and placement in the autosampler for 24 hours in comparison with freshly extracted QCs; however, there were signs of degradation including distorted peaks and appearance of unknown peaks after storage for 48 hours in the autosampler. The results for long-term stability were obtained by comparing the concentration of the QCs stored at -20°C for 6 months with those obtained before the storage period. As shown in Table 3, there was no significant change in the concentration of any of the analytes. The stock solution stability test was performed by placing an aliquot of the stock solution at room temperature for a period of 6 hours and comparing it with freshly prepared stock. The change in the concentrations of MPAG, AcMPAG, and MPA were $2.24 \pm 1.36\%$, $0.31 \pm 0.89\%$, and $1.0 \pm 0.43\%$. This indicates that the stock solutions were stable up to 6 hours at room temperature.

Figure 4 depicts concentration–time profiles for MPA, AcMPAG, and MPAG from 3 representative stable kidney transplant recipients in plasma samples collected at specified time intervals over a 12-hour period after drug administration. Patients 1 and 3 were on 500 mg MMF dose twice a day, and patient 2 was on 1000 mg MMF dose twice daily. The 3 patients were selected as representative only to show the range of

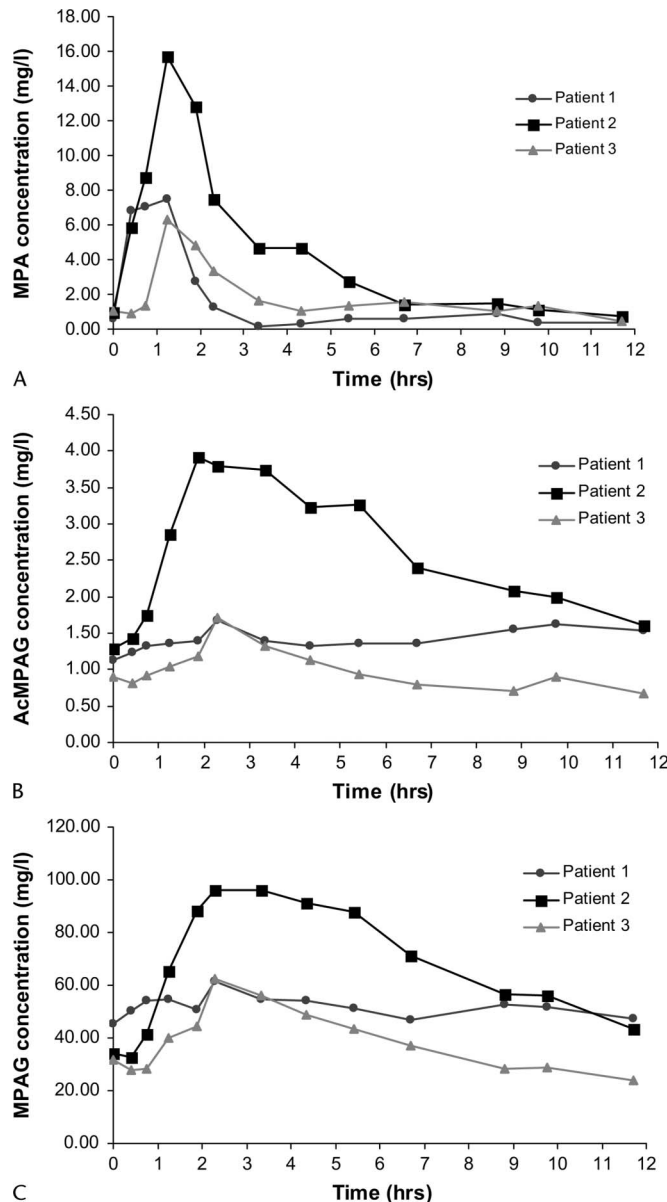


FIGURE 4. A, Representative concentration–time profile for MPA from 3 kidney transplant recipients on mycophenolate mofetil therapy over a period of 12 hours after drug administration. (Patients 1 and 3 were on 500 mg mycophenolate mofetil twice daily, and patient 2 was on 1000 mg twice daily.) B, Representative concentration–time profile for AcMPAG from 3 kidney transplant recipients on mycophenolate mofetil therapy over a period of 12 hours after drug administration. C, Representative concentration–time profile for MPAG from 3 kidney transplant recipients on mycophenolate mofetil therapy over a period of 12 hours after drug administration.

expected concentrations rather than for their specific pharmacokinetic characteristics. In addition, the 23 patients included in this study provided a total of 293 plasma samples with concentrations of MPA, MPAG, and AcMPAG, all within the range of quantification for the assay (Table 4), in which the

TABLE 4. Plasma Concentrations of 23 Kidney Transplant Recipients in a Clinical Pharmacokinetics Study Determined Using the HPLC-UV Method Described

Analyte	C _{max} (mg/L)	C _{min} Morning* (mg/L)	C _{min} Evening† (mg/L)
MPA			
Mean ± SD	11.77 ± 9.43	2.24 ± 3.11	2.07 ± 2.08
Range	2.70–40.27	0.31–14.76	0.30–8.71
MPAG			
Mean ± SD	88.15 ± 46.49	55.44 ± 29.55	51.11 ± 25.98
Range	29.67–212.15	17.51–138.30	19.34–120.71
AcMPAG			
Mean ± SD	3.01 ± 1.73	1.42 ± 0.74	1.73 ± 1.04
Range	0.75–7.77	0.45–3.20	0.60–4.42

*C_{min} Morning is trough concentration obtained at 8:00 AM before the morning MMF dose.

†C_{min} Evening is trough concentration obtained at 8:00 PM 12 hours after the morning MMF dose.

concentration–time profile of 17 patients showed signs of enterohepatic recirculation.

DISCUSSION

We report a modified version of an earlier published HPLC-UV method¹⁶ for simultaneous quantification of MPA and its 2 metabolites MPAG and AcMPAG. Our original method was developed in 1998 and has been used for a number of years in 3 laboratories (Dr. David Holt's Laboratory in London UK, Late Dr. Andrew Trull's Laboratory in Cambridge UK, and Dr. Fatemeh Akhlaghi's Laboratory in the University of Rhode Island) and has proven to be a robust and highly reproducible assay. We have now modified the assay to also measure AcMPAG concentrations, improved its limit of quantification for MPA and MPAG, comprehensively validated the method according to the FDA guidelines,¹⁷ and report a range of expected concentrations for the 3 analytes in kidney transplant recipients.

Therapeutic monitoring of MPA has gained considerable clinical importance,¹⁹ and monitoring MPAG and AcMPAG concentrations along with MPA may also help to indicate the degree of immunosuppressive activity or adverse effects of the drug.²⁰ Quantitative determination of both MPA metabolites is important because MPAG is known to undergo enterohepatic recirculation and reenter the systemic circulation as MPA.¹ Also MPAG is known to accumulate in the body in renally impaired patients and displaces MPA from its albumin binding sites, resulting in higher unbound MPA concentrations.^{21,22} AcMPAG, on the other hand, is believed to have immunosuppressive activity⁶ and to possess proinflammatory effects in the gut. The concentration of AcMPAG may therefore associate to a greater extent with the occurrence of diarrhea^{23,24} and leukopenia.⁷ Availability of a sensitive, specific, and robust analytical method for the analysis of all 3 analytes in a single run is therefore warranted to facilitate the therapeutic drug monitoring and pharmacokinetic studies of MPA.

In the first assay reported for the quantification of AcMPAG in plasma, Shipkova et al¹⁴ have observed significant degradation of AcMPAG in nonacidified plasma samples as characterized by changes in plasma concentration of the analytes and

distorted peak shapes. In contrast to this observation, and similar to the method described by Khoschsorur and Erwa,¹⁵ we have found that even without prior acidification of plasma samples, AcMPAG in plasma extracts is stable in the autosampler for up to 24 hours. We speculate that this variation could be related to the differences in the protein precipitation reagents used; for example, the method by Shipkova et al¹⁴ uses sodium tungstate and perchloric acid, whereas the method by Khoschsorur and Erwa¹⁵ and our method use only phosphoric acid in combination with acetonitrile or methanol, respectively. In the light of the current study, we do not see a need for prior acidification of plasma sample to prevent degradation of AcMPAG to MPA. In addition, all the results presented in Table 3 are based on double injections of each QC standard in triplicates. In the case of MPA QC1 during autosampler stability and AcMPAG QC1 during short-term stability, where the mean ± SD falls slightly outside the recommended range, only 1 of the 2 injections gave a slightly inaccurate concentration (118% and 83% respectively), but the other injection gave a result within a few percent of the expected concentration. We believe the inaccurate result of a single injection, is originated from experimental or instrumental errors rather than an inherent problem in the stability of the compounds in question.

The method described is highly specific, robust, and has adequate degree of sensitivity to be able to analyze samples for pharmacokinetic studies or therapeutic drug monitoring. The possibility of interference from drugs that are normally coadministered with MMF was evaluated for interference with the chromatographic retention time of MPA, AcMPAG, and MPAG. However, most of these drugs did not pass into the final extract of the SPE procedure. Some of the drugs that were extracted showed peaks that had retention times different from the analytes and IS of interest. In addition, plasma samples from 21 renal transplant patients that were not on MPA therapy were analyzed to evaluate the possibility of interference from endogenous or exogenous compounds. None of the samples showed any peaks at the retention times of the analytes.

The method has a LLOQ of 0.2, 0.5, and 2 mg/L for MPA, AcMPAG, and MPAG, respectively, and actually has an on-column concentration of 0.06, 0.16, and 0.66 mg/L

respectively for MPA, AcMPAG, and MPAG because of the dilution factor (1:3) applied to the final elution step in the extraction procedure. It must be noted that in the current method we have set our detection wavelength at 254 nm rather than 214 nm used by the other 2 methods^{14,15} because 254 nm provided a cleaner baseline and more reproducible results. During the initial method development stage, we observed that switching from 214 to 254 nm reduced the peak height of the analytes approximately 13%–17% but also reduced the baseline noise by an average of 87%; therefore, 254 nm was selected as the ultraviolet wavelength of choice for the method.

The current method uses a simple SPE procedure and does not require additional centrifugation steps, which results in a simpler overall extraction procedure. It also uses 100 μ L of plasma sample, which is less than 200–250 μ L, the volume used by some of the other methods.^{11,12} Moreover, the method has been validated according to the Guidelines for Bioanalytical Method Validation¹⁷ set by the FDA, which require validation data for at least 6 calibration curves, but in the current method, data for 10 calibration curves have been presented. This analytical method has been successfully used in our laboratory to determine the plasma concentrations of total MPA, AcMPAG, and MPAG in 23 kidney transplant recipients during a steady-state clinical pharmacokinetics study.

CONCLUSIONS

It has become clear in the recent years that plasma monitoring of all 3 analytes, MPA, AcMPAG, and MPAG, may prove beneficial for the long-term management of patients receiving mycophenolic acid. The analytic method described, which is an improved version of an already simple and reproducible method, has an adequate degree of flexibility, accuracy, and robustness for everyday monitoring of all 3 analytes and/or for analyzing samples obtained during pharmacokinetic studies of mycophenolic acid.

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