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Quantitation of mycophenolic acid and metabolites by UPLC-MS/MS in renal transplant patients

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Abstract

Mycophenolate mofetil (MMF) and enteric-coated mycophenolate sodium (EC-MPS), specific inhibitors of lymphocyte proliferation, are commonly used as adjuvant therapy with calcineurin inhibitor agents after kidney transplantation. After administration, MMF and EC-MPS are hydrolyzed to mycophenolic acid (MPA), the active form of the drug, which must be monitored due to its narrow therapeutic window, drug–drug interactions, and large intra- and inter-individual pharmacokinetic variability despite a fixed dose. Monitoring plasma MPA level is recommended to maintain the drug within the therapeutic window, optimize its efficacy, and minimize side effects. This study aims to develop a method for quantifying MPA and its major metabolites (mycophenolic acid glucuronide [MPAG]) using on-line solid phase extraction (SPE) coupled with an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) in kidney transplant patients. The linearity of MPA and MPAG were 0.3–13.6 µg/mL and 2.6–232.9 µg/mL, respectively ($r^2 > 0.999$). The relative error of accuracy was <15%. The within-run and between-run imprecision was <5.8%. No carryover, ion suppression, or ion enhancement were observed. This method was used to analysis of 351 plasma samples from renal transplant patients after MMF or EC-MPS using this method showed large pharmacokinetic variability between patients. Analysis of the same samples by immunoassay showed a large positive bias compared with our validated UPLC-MS/MS method, averaging 15.1%. These results suggest that this UPLC-MS/MS method is more effective than immunoassay for quantitation of MPA and its metabolites in clinical samples.

Keywords: MPA, Mycophenolic acid, Renal transplant, UPLC-MS/MS

1. Introduction

Co-administration of an anti-proliferative immunosuppressive drug and calcineurin inhibitors (tacrolimus or cyclosporine A) is the recommended treatment for patients after kidney transplantation [1]. Mycophenolate mofetil (MMF) and enteric-coated mycophenolate sodium (EC-MPS) are both anti-proliferative immunosuppressant drugs approved by the Food and Drug Administration (FDA) in 1995 and 2004, respectively [2]. These drugs inhibit T and B lymphocyte proliferation. MMF and EC-MPS are prodrugs, with MMF rapidly hydrolyzed into the active drug mycophenolic acid (MPA) after administration. In the intestine EC-MPS also is converted to active MPA. MPA is an inhibitor of inosine monophosphate dehydrogenase (IMPDH), a rate-limiting enzyme in

the de novo synthesis of guanosine nucleotides. MPA is metabolized by uridine diphosphate glucuronosyltransferase (UTG) to phenolic mycophenolic acid glucuronide (MPAG) in the liver, kidney, and intestine and excreted in urine. The inactive metabolite MPAG can be converted back to MPA by β -glucuronidase during enterohepatic recirculation, leading to a second plasma concentration peak that can increase its toxicity. MPA is further metabolized into two minor metabolites, acyl glucuronide (AcMPAG) and phenolic glucoside of MPA [3]. The elimination half-life of MPA average 18 h, MPAG and AcMPAG are the metabolites of MPA. Studies of MPA pharmacokinetics has shown that exposure correlated poorly with the dose of the drug, and many patients on standard fixed dosing have sub-therapeutic concentration of MPA. MPA exhibits significant intra- and inter-patient variation in plasma

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concentration, undergoes drug–drug interactions, and has a narrow therapeutic window. It has therefore been suggested that monitoring plasma concentrations of MPA is useful to overcome the variable, especially in patients after kidney transplantation [4,5]. Assays for monitoring MPA include immunoassays [6,7], high-performance liquid chromatography with ultraviolet detection (HPLC-UV) [8], and LC-MS/MS [9–13]. The immunoassays approved by the FDA in 2001 are fast and easy to carry out but can over-estimate MPA concentrations due to nonspecific cross-reactions between antibodies and MPAG or AcMPAG [8,12]. HPLC-UV and LC-MS/MS are more specific than immunoassays but are still not popular because the devices needed are highly technical and expensive. On-line SPE combines sample preparation with chromatography, providing cleaner samples and allowing for batch processing. The combination of on-line SPE with UPLC-MS/MS may provide rapid and easy analysis for use in the clinical laboratory. This study aims to develop and validated a simple method for clinical laboratory measurement of MPA, MPAG, and AcMPAG using on-line solid phase extraction (SPE) coupled with UPLC-MS/MS. Method verification was conducted according to Clinical Laboratory Improvement Amendments (CLIA) guidelines.

2. Materials and methods

2.1. Reagents and chemicals

LC-MS grade methanol was purchased from J.T Baker (Phillipsburg, NJ, USA). Formic acid and ammonium formate were from Sigma–Aldrich (St. Louis, MO, US). Water was prepared in-house using a Millipore Milli-Q Advantage A10 purification system (Darmstadt, Germany). Liquid MPA and MPAG were purchased from Cerilliant corporation (Round Rock, Texas, USA). MPA-d3, MPAG-d3 and AcMPAG-d3 were from Toronto Research Chemicals (Toronto, Canada). The structure of MAP, MPAG and AcMPAG are shown in Fig. 1. The Chromsystems 6 PLUS1 multilevel calibrator mycophenolic acid/glucuronide in plasma/serum calibration kit (Munich, Germany) was used to evaluate accuracy. For immunoassays, the mycophenolic acid Flex reagent cartridge, calibrators, and controls were all purchased from Siemens Healthcare Diagnostics (Frimley, UK).

2.2. Plasma samples

A total of 105 renal transplant patients were enrolled from March 2015 to January 2017, and 351 plasma samples were collected under the Chang

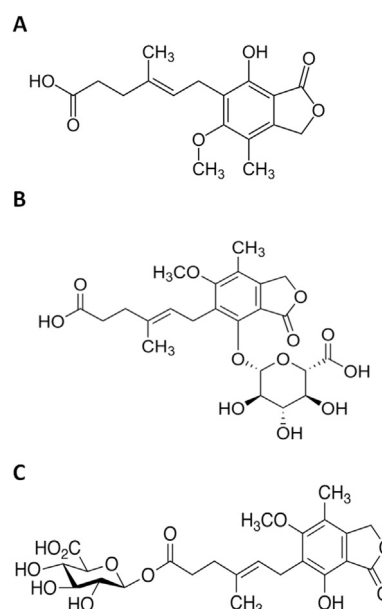


Fig. 1. Structure of MPA (A), MPAG (B), and AcMPAG (C).

Gung Medical Foundation Institutional Review Board (IRB) Protocol# 103-6727 B.

2.3. Calibrators and controls

Stock solutions of MPA (1 mg/mL) and MPAG (1 mg/mL) were prepared in acetonitrile and stored at -70°C . Using these stock solutions, a calibration curve was made using pooled drug-free plasma at concentrations of 0.0, 0.5, 1.0, 2.0, 5.0, 7.5, 10.0 $\mu\text{g/mL}$ MPA and 0.0, 10.0, 20.0, 40.0, 100.0, 150.0, 200.0 $\mu\text{g/mL}$ MPAG. Calibration curves were established by plotting the peak area ratios of the analytes to stable isotope labeled versus the analyte concentrations with 1/X weighted linear regression analysis [14]. Controls contained MPA at 2.0 and 5.0 $\mu\text{g/mL}$ and MPAG at 40.0 $\mu\text{g/mL}$ and 120.0 $\mu\text{g/mL}$.

2.4. Internals standards and precipitation reagents

Stock internal standard solutions of MPA-d3 (1 mg/mL), MPAG-d3 (1 mg/mL) and AcMPAG-d3 (1 mg/mL) were prepared in methanol and stored at -70°C . The protein precipitation reagent was 100% methanol and contained MPA-d3, MPAG-d3, and AcMPAG-d3 at 50 ng/mL, 1,000 ng/mL, and 100 ng/mL, respectively.

2.5. Sample preparation

Calibrators and controls were prepared in drug-free plasma to compensate the matrix effect and treated in the same way as patient samples. A 10- μL

aliquot of calibrator, control, or sample was transferred into a 1.5 mL centrifuge tube containing 500 μ L of protein precipitation reagent. Each tube was vortexed and then centrifuged at 12,000 rpm for 15 min. To 1 mL of water was added 100 μ L supernatant, and the solution was directly injected into UPLC-MS/MS.

2.6. UPLC-MS/MS instrument and condition

We used the Waters Acquity Ultra Performance Liquid Chromatography connected to the Waters Xevo TQ-S mass spectrometry (Manchester, UK) for quantitation and MassLynx software for data acquisition. The Waters Direct Connect HP Xbridge TM C18 (2.1×30 mm; 10 μ m) (Wexford, Ireland) was used for on-line SPE, and the Waters ACQUITY UPLC BEH C18 (2.1×50 mm; 1.7 μ m) (Wexford, Ireland) was used for analytical separation. The on-line SPE and column were maintained at a temperature of 65 °C. Mobile phase A contained 5 mM ammonium formate and 0.03% formic acid, and mobile phase B contained methanol. The flow rate of the on-line SPE was set at 1.0 mL/min. The gradient program was 25% B for 1 min, followed by an increase to 95% for 2 min. The flow rate of the analytical column was set at 0.5 mL/min. The gradient program was 40% B for 1 min, followed by an increase to 95% for 1.6 min. The injection volume was 5 μ L, and the total run time was 6 min. The mass spectrometry operated with multiple reaction monitoring (MRM) and positive electrospray ionization. For all compounds, the optimized mass parameters were as follows: capillary voltage, 0.6 kV; desolvation temperature, 400 °C; desolvation gas flow, 800 L/h; cone gas flow, 150 L/h; and source temperature, 120 °C. The MRM transitions, cone voltages, collision energies, and dwell time used for the analysis of MPA and its metabolites are presented in Table 1.

2.7. Method verification

2.7.1. Linearity

Plasma was spiked with MPA or MPAG at 11 concentrations (MPA: 0.1, 1.4, 2.7, 4.1, 5.4, 6.7, 8.0, 9.3,

10.6, 12.0, 13.3 μ g/mL; MPAG: 5.3, 28.3, 51.4, 74.4, 95.7, 120.5, 143.6, 166.6, 189.7, 212.7, 235.7 μ g/mL). The linearity of the assay was determined for each series in duplicate (two consecutive injections of the same concentration), with calculation of the coefficient of determination (R^2) and bias. A calibration kit from Chromsystems (Grafelfing, Germany) was used to evaluate the accuracy of the measurements (MPA: 0.9, 1.6, 3.2, 3.8, 6.6, 9.2 μ g/mL; MPAG: 16.2, 35.1, 70.1, 88.0, 157.0, 222.0 μ g/mL), and bias should be <15%.

2.7.2. Imprecision

The within-run and between-run imprecision was determined by analyzing low and high concentrations 20 times a day (within-run imprecision) and duplicating (two consecutive injections of the same concentration) on 10 different days (between-run imprecision). The imprecision data are presented as the standard deviation (SD) and coefficient of variation (CV%).

2.7.3. Lower limit of quantitation (LLOQ)

Decreasing concentration samples for 8 replicates were analyzed on 5 different days. At the lowest concentration, the CV, accuracy, and S/N ratio were <20%, <20%, and >10, respectively.

2.7.4. Carryover

Plasma was spiked with high and low concentrations of MPA (High: MPA, 12.1 μ g/mL; MPAG, 234.7 μ g/mL) (Low: MPA, 2.0 μ g/mL; MPAG, 40.0 μ g/mL). The carryover was analyzed and determined in the following order: L1, L2, L3, H1, H2, L4, H3, H4, L5, L6, L7, L8, H5, H6, L9, H7, H8, L10, H8, H9, L11. The influence of low to high concentration was calculated.

2.7.5. Ion suppression

The post-column infusion method was used [15]. A 100-ng/mL mixture of standards (MPA, MPAG, and AcMPAG) along with 10 different drug-free plasma samples were infused into the MS detector via the T-valve. Chromatography data were compared with blank solvent, and the ion suppression and enhancement in the peak regions were evaluated.

Table 1. UPLC-MS/MS MRM transitions, dwell time, and cone and collision voltages used for analyzing MPA and its metabolites.

Analyte name	Quantifier	Qualifier	Collision energy (eV)	Dwell time (s)	Cone (V)
MPA	321.18 \rightarrow 207.04	321.18 \rightarrow 275.11	24/14	0.003	8
MPA-d3	324.14 \rightarrow 210.11		22	0.003	2
AcMPAG	519.18 \rightarrow 343.05	519.18 \rightarrow 228.99	20/32	0.1	8
AcMPAG-d3	522.18 \rightarrow 346.05		32	0.1	8
MPAG	519.18 \rightarrow 343.05	519.18 \rightarrow 228.99	18/30	0.05	22
MPAG-d3	522.18 \rightarrow 346.05		18	0.05	22

2.7.6. Interference

The effects of hemolysis, triglycerides, and icteric on the concentrations of MPA and MPAG were evaluated.

2.8. Immunoassay

The mycophenolic acid assay was performed using a Dimension EXL analyzer obtained from Siemens Healthcare Diagnostics (Frimley, UK). The functional sensitivity is claimed to be 0.2 µg/mL in the package insert, but the assay LOQ is verified to be 0.55 µg/mL in our laboratory.

2.9. Method comparison

Passing–Bablok regression analysis was used to assess the correlation between the two methods, and a Bland–Altman plot was used to evaluate the difference between the two methods. The data were statistically analyzed using Excel.

3. Results

3.1. UPLC-MS/MS method

The UPLC-MS/MS method used to quantify MPA, MPAG, and AcMPAG together is shown in Fig. 2. The sample preparation time was less than 30 min, and the total run time for UPLC-MS/MS was 6 min. The shapes of the MPA and MPAG peaks on the chromatogram were sharp. MPAG was the first eluting analyte, AcMPAG was the second, and MPA was the last (Fig. 2).

3.2. Method verification

The linear ranges of MPA and MPAG were 0.1–13.6 µg/mL and 0.8–232.9 µg/mL, respectively, and the coefficient of determination (R^2) was >0.999 (Fig. 1 in Supplementary data). Data regarding the accuracy of the assay are shown in Table 2. The bias of MPA and MPAG were $<15\%$ compared to commercial standards. The LLOQ was 0.25 µg/mL for MPA and 2.61 µg/mL for MPAG. The quantitative range was 0.3–13.6 µg/mL for MPA and 2.6–232.9 µg/mL for MPAG. The method showed good precision and the within-run and between-run for MPA and MPAG were $<5.8\%$ (Table 3). No interference was observed with hemoglobin at 300 mg/dL, triglycerides at 1.119 mg/dL, or bilirubin at 6.25 mg/dL. The carryover test showed that low concentration might not be affected by MPA concentration at 12.1 µg/mL or MPAG concentration at 234.7 µg/mL (Table 1 in Supplementary data). Ion suppression was evaluated by post-column infusion, and no ion suppression or enhancement was shown in the peak region (Fig. 3).

3.3. Comparison between immunoassay and UPLC-MS/MS results

We compared the immunoassay and UPLC-MS/MS method by analyzing 351 samples. Passing–Bablok regression analysis showed good linearity between the two methods (Fig. 4A), with a Pearson coefficient of $r = 0.99$ for MPA. Bland–Altman regression analysis showed a significant positive bias of 15.1% (95% CI, -15.3 – 50.0%) (Fig. 4B).

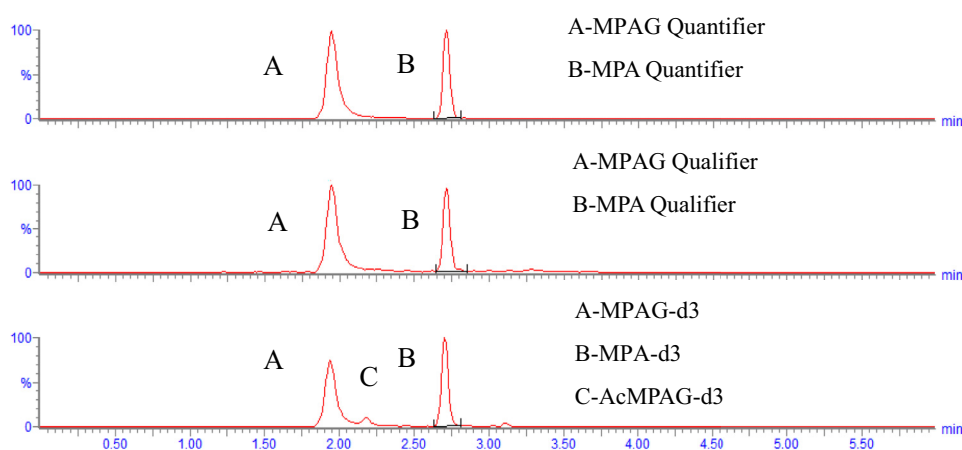


Fig. 2. Chromatography of patient samples with added MPA (3.5 µg/mL) and MPAG (113.7 µg/mL). AcMPAG-d3 was added to monitor the presence/absence of the unstable metabolite AcMPAG.

Table 2. Accuracy of determining concentrations of the analyzed compounds.

		n = 12					n = 12		
		Mean ($\mu\text{g/mL}$)	Target value ($\mu\text{g/mL}$)	Bias (%)			Mean ($\mu\text{g/mL}$)	Target value ($\mu\text{g/mL}$)	Bias (%)
MPA	level 1	1.1	0.9	13.8	MPAG		14.9	16.2	–8.0
	level 2	1.7	1.6	8.0			30.7	35.1	–12.4
	level 3	3.4	3.2	6.5			61.7	70.1	–11.9
	level 4	4.1	3.8	8.5			77.4	88.0	–12.0
	level 5	7.3	6.6	11.1			133.8	157.0	–14.8
	level 6	10.3	9.2	11.4			192.8	222.0	–13.0

3.4. Assay method applied to patients treated with MPA

We used the verified method to analyze 351 plasma samples of patients who were administrated 0–1,500 mg/mL MMF or EC-MPS. We observed plasma concentrations of <0.3–17.8 $\mu\text{g/mL}$ MPA and <2.6–192.8 $\mu\text{g/mL}$ MPAG. In 120 samples co-treated with MPA and tacrolimus, the plasma MPA concentration range was <0.3–13.2 $\mu\text{g/mL}$; in 36 samples co-treated with MPA and cyclosporine A, the plasma MPA concentration range was <0.3–15.8 $\mu\text{g/mL}$. No correlation was observed between dose and concentration, and the results showed large inter- and intra-individual variation.

4. Discussion

The data show that we successfully established a robust assay that uses on-line SPE coupled with UPLC-MS/MS to quantify MPA, MPAG, and monitor the presence of AcMPAG together in one sample. According to a previous study, AcMPAG is a metabolite of MPA, and the structure of AcMPAG is unstable [16]. The AcMPAG metabolite concentration was relatively low and less stable than that of MPA and MPAG. MPA is primarily glucuronidated to the major inactive MPA phenolic glucuronide metabolite (MPAG) and the minor active MPA acyl glucuronide metabolite (AcMPAG) by uridine diphosphate glucuronosyl transferase (UGT) at the phenolic hydroxyl group and the acyl group, respectively. AcMPAG seems to be associated with the gastrointestinal toxicity of MPA. However, it is

controversial whether AcMPAG monitoring is helpful for predicting efficacy and toxicity in MPA therapy, because only one systematic study has assessed the association between AcMPAG plasma concentration and the incidence of diarrhea in patients on MMF [17]. We added AcMPAG-d3 stable-isotope-labeled internal standards as done in some clinical laboratories to monitor the presence of AcMPAG and provide more extensive results than just quantification of MPA and MPAG. Two concentrations of spiked control samples were used to monitor the stability of MPA and MPAG. Our data show that MPA and MPAG are stable for over 6 months when stored at -70°C . The assay requires only 10 μL of plasma and uses a simple protein precipitation protocol. We observed that MPA, MPAG, and AcMPAG eluted at 2.71, 1.94, and 2.18 min, respectively, for a total run time of 6 min.

Our protocol uses an automatic sample-extraction method (on-line SPE) coupled with an analytical column to isolate MPA, MPAG, and an added AcMPAG internal standard from the same sample. The advantages of our method include a lower sample volume, automatic extraction procedure, minimal ion suppression, and good performance.

The method was verified according to the clinical laboratory guidelines: Clinical and Laboratory Standards Institute (CLSI) guideline C62-A Liquid Chromatography – Mass Spectrometry Methods; Approved guideline [14]. The verification data demonstrate good linearity, low imprecision, and no carryover or matrix effects. We used commercially available calibration standards to evaluate the accuracy of the method. The guideline state that an

Table 3. UPLC-MS/MS within-run and between-run data for each control level.

Analyte		UPLC-MS/MS Within-run (n = 20)				UPLC-MS/MS Between-run (n = 28)			
		Mean ($\mu\text{g/mL}$)	SD ($\mu\text{g/mL}$)	CV (%)	Recovery (%)	Mean ($\mu\text{g/mL}$)	SD ($\mu\text{g/mL}$)	CV (%)	Recovery (%)
MPA	QC level 1	2.1	0.1	4.8	105	2.1	0.1	4.8	105
	QC level 2	4.9	0.2	3.2	98	5.2	0.3	5.8	104
MPAG	QC level 1	40.6	0.5	1.2	101	41	0.6	1.6	103
	QC level 2	118	1.1	0.9	98	123.6	3.4	2.8	103

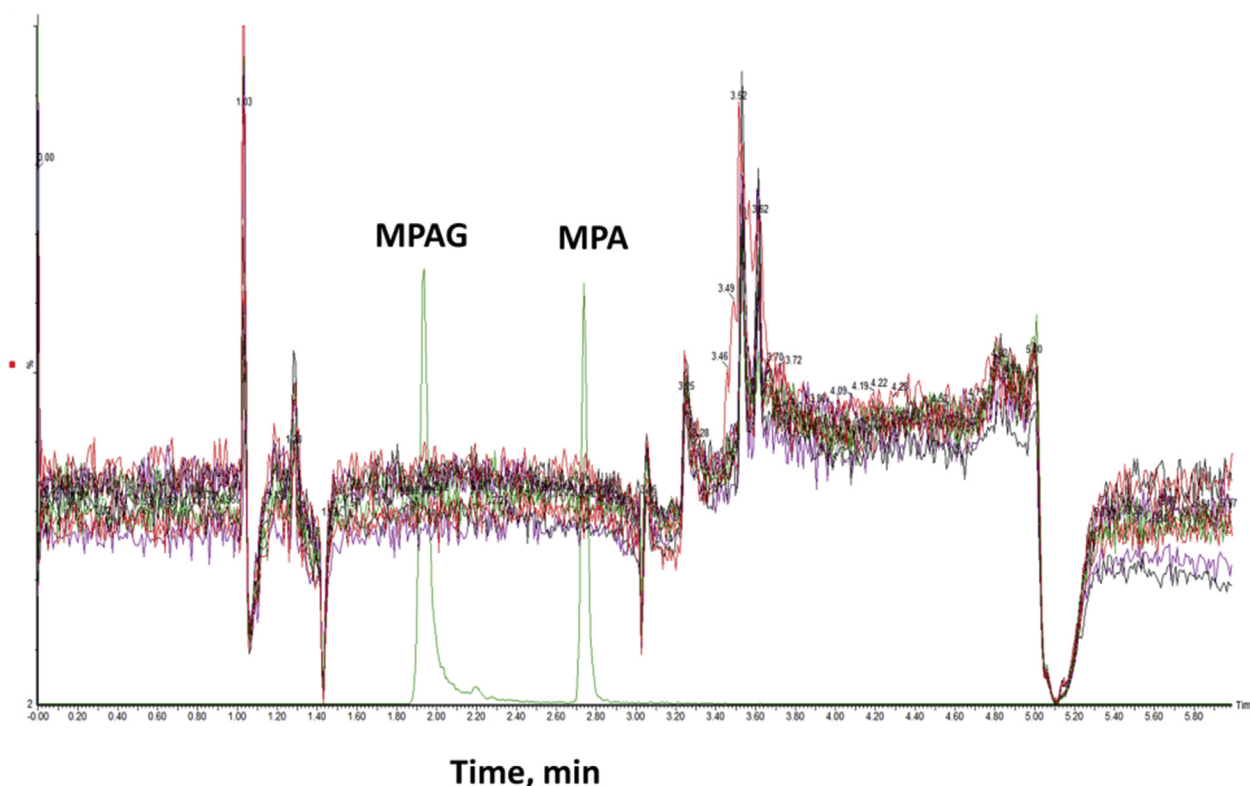


Fig. 3. Ion suppression profile performed with a post-column flow injection of 100 ng/mL MPA and MPAG into the UPLC eluate of drug-free samples from 10 healthy individuals.

acceptable bias is 15%. The data show a mean bias of 10% for MPA and -12% for MPAG. These results suggest that the discrepancy between 6.5% and 13.8% of MPA and between -8% and -14.8% of MPAG were caused by the use of different standards. The calibrators from Chromsystems were lyophilized and needed to be reconstituted before use. The 7-point calibration curve was prepared from stock liquid standard solutions that are certified reference materials for determining the accuracy of MPA and MPAG concentrations. Since the MPA/MPAG concentration is not standardized, we then used proficiency-testing samples to evaluate the consensus of the MPA results. Using proficiency-testing samples from the College of American Pathologists to monitor MPA concentrations, the bias was $<3.5\%$ of the mean value of the peer group. These samples do not contain MPAG for evaluation.

In-source fragmentation of MPAG and AcMPAG was found in our assay and also has been reported in other studies [18,19]. If MPA is not separated from MPAG and AcMPAG, the resulting MPA concentrations are affected. In our method, we chromatographically separated MPA, and MPAG, and

AcMPAG to yield baseline resolution of MPA to more accurately determine the MPA, MPAG, and AcMPAG concentrations.

Passing–Bablok regression analysis showed good linearity between the immunoassay and our method. However, the Bland–Altman regression analysis showed a significant positive bias of the immunoassay in 351 clinical plasma samples. Previous studies have shown that immunoassays may be affected by the metabolite MPAG or AcMPAG, overestimating the plasma MPA concentration by 26.25% (95% CI, 21.43–31.07%) [7]. We found that the mean MPAG/MPA ratio was 31.9 when the positive bias of the immunoassay was $>15.1\%$ and 22.7 when the positive bias was $<15.1\%$ ($p < 0.05$). These data show that the presence of MPAG affects quantitation of MPA in the immunoassays.

According to the Tietz Textbook of Clinical Chemistry and Molecular Diagnostics [20], the therapeutic range of MPA is 1.0–3.5 $\mu\text{g/mL}$ in patients administered MPA with cyclosporine A and 1.9–4.0 $\mu\text{g/mL}$ in those administered MPA with tacrolimus. In our analysis MPA levels in 351 renal transplant patients, a plasma concentration below the therapeutic range was observed in only 46.7% of

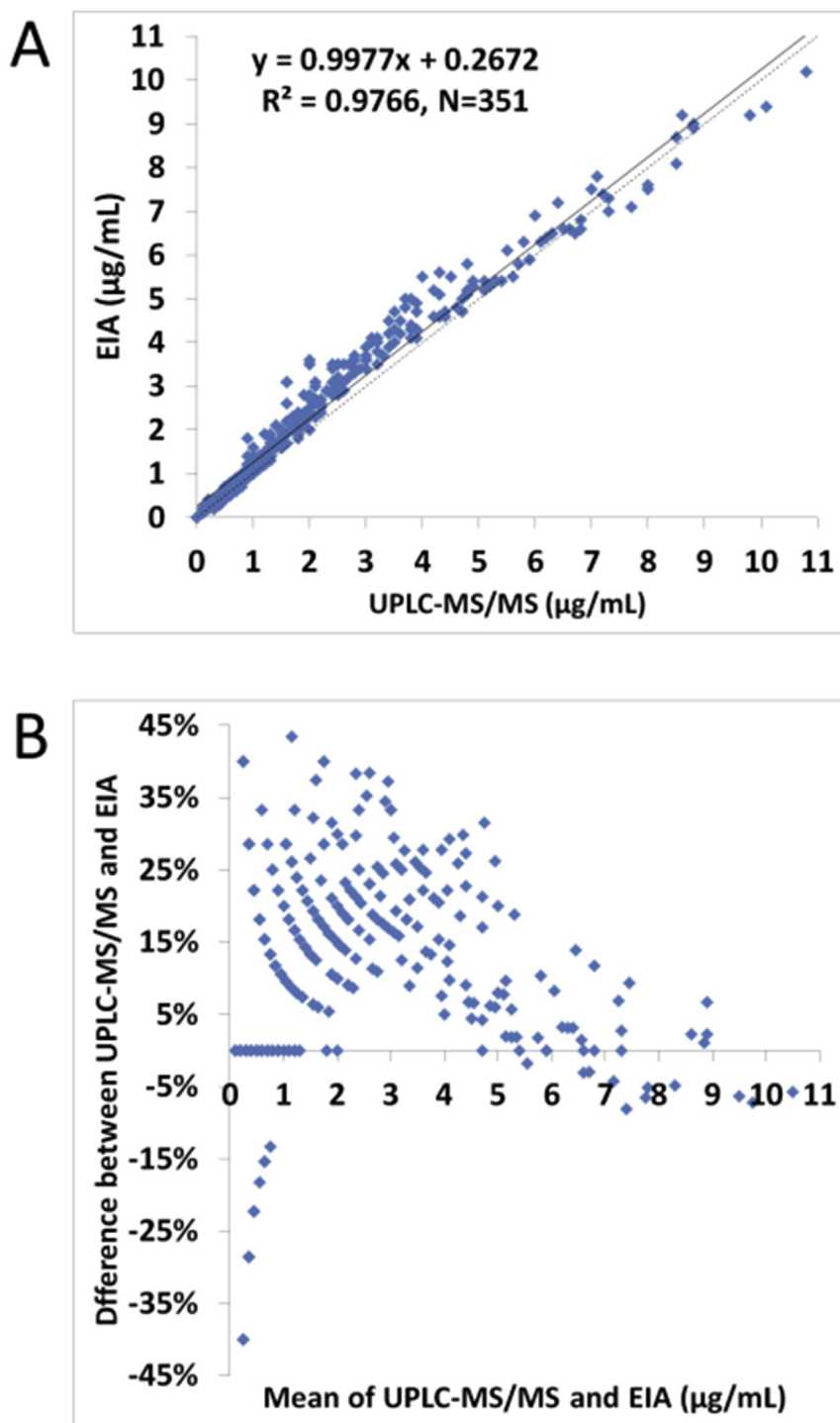


Fig. 4. Comparison of results between UPLC-MS/MS and immunoassay. (A) Passing–Bablok regression analysis of immunoassay vs. UPLC-MS/MS. (B) Bland–Altman plot of immunoassay vs. UPLC-MS/MS.

those co-treated with MPA and tacrolimus and 19.4% of those co-treated with MPA and cyclosporine A.

Studies of MPA pharmacokinetics have shown that exposure correlates poorly with the dose of the drug, and many patients on standard fixed dosing have

subtherapeutic concentrations of MPA [21]. Therefore, monitoring plasma concentrations of MPA is suggested to be useful to compensate for the variable pharmacokinetic behavior of this drug. According to the prescribing information, there are no significant sex or age differences in pharmacokinetics. In our

study, we demonstrated advantages of using the UPLC-MS/MS platform to quantify MPA and MPAG. At the same time, we compared this method to a commercially available immunoassay and evaluated the bias between the two platforms. The UPLC-MS/MS platform provided more precise data than did the immunoassay and could be useful in the clinical setting.

5. Conclusion

Monitoring plasma concentrations of MPA is critically important to optimizing patient drug dosage. UPLC-MS/MS is more accurate than immunoassay for quantifying plasma MPA. This verified method provides greater sensitivity and

specificity for effective monitoring of MPA and its metabolites in clinical laboratories.

Conflicts of interest

The authors declare that they have no conflict of interests.

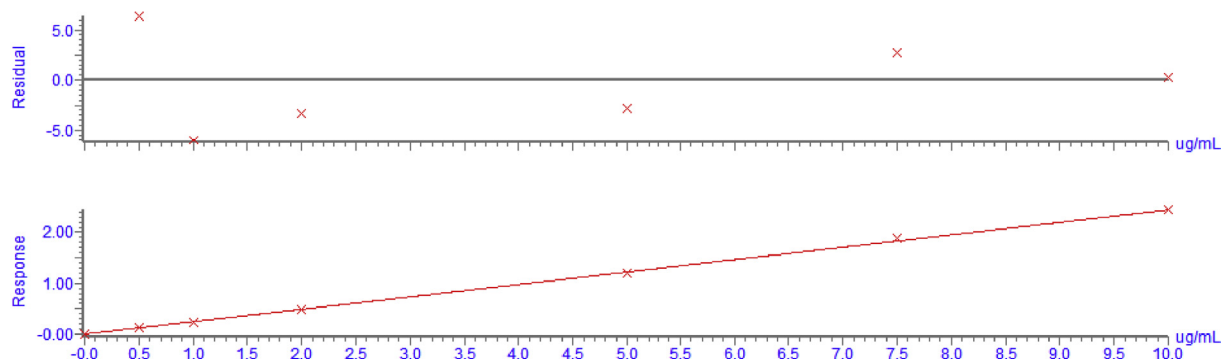
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Appendix. Supplementary data

A

Compound name: Mycophenolic acid
Correlation coefficient: $r = 0.999546$, $r^2 = 0.999092$
Calibration curve: $0.24367 \times x + 0.00211412$
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: $1/x$, Axis trans: None



B

Compound name: MPAG
Correlation coefficient: $r = 0.999740$, $r^2 = 0.999480$
Calibration curve: $0.0208547 \times x + 0.00607706$
Response type: Internal Std (Ref 4), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: $1/x$, Axis trans: None

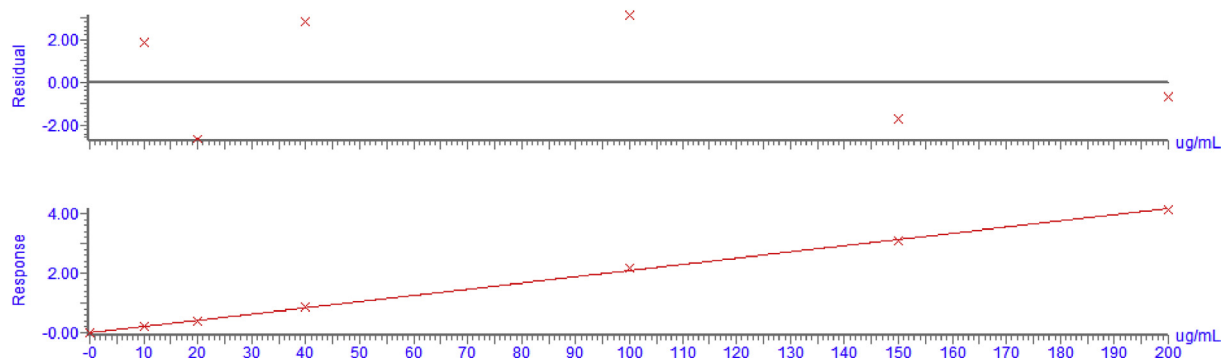


Fig.1. Standard residual and calibration curve for MPA and MPAG. (A) MPA. (B) MPAG.

Table 1. Carryover results for MPA and MPAG. (A) MPA. (B) MPAG.

A				
MPA	Concentration (µg/mL)	Low-Low Result	High-Low Result	High Result
L1	1.9			
L2	2.4	2.4		
L3	2.0	2.0		
H1	11.4			11.4
H2	12.6			12.6
L4	2.3		2.3	
H3	11.9			11.9
H4	11.9			11.9
L5	2.2		2.2	
L6	2.1	2.1		
L7	2.0	2.0		
L8	2.4	2.4		
H5	11.8			11.8
H6	11.8			11.8
L9	2.2		2.2	
H7	13.1			
H8	12.3			
L10	2.2		2.2	
H9	12.3			12.3
H10	12.1			12.1
L11	2.2		2.2	
Mean		2.2	2.2	12.1
SD		0.2	0.0	0.5
High-Low Mean		2.2		
Low-Low Mean		2.2		
Carryver		0.0		
Error Limit		0.6		
B				
MPAG	Concentration (µg/mL)	Low-Low Result	High-Low Result	High Result
L1	41.6			
L2	41.6	41.6		
L3	41.1	41.1		
H1	230.0			230.0
H2	235.2			235.2
LA	41.4		41.4	
H3	236.1			236.1
H4	240.6			240.6
L5	41.5		41.5	
L6	40.3	40.3		
L7	40.9	40.9		
L8	40.3	40.3		
H5	231.8			231.8
H6	237.1			237.1
L9	41.2		41.2	
H7	236.7			236.7
H8	234.0			234.0
L10	41.0		41.0	
H9	231.4			231.4
H10	233.7			233.7
L11	41.7		41.7	
Mean		40.8	41.4	234.7
SD		0.6	0.3	3.2
High-Low Mean		41.4		
Low-Low Mean		40.8		
Carryver		0.5		
Error Limit		1.7		

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