ORIGINAL ARTICLE

Development and validation of an ultra-performance liquid chromatography mass spectrometry/mass spectrometry method for simultaneous quantification of total and free mycophenolic acid and its metabolites in human plasma

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ABSTRACT

A reliable method has been validated using ultra-performance liquid chromatography mass spectrometry (MS)/MS for simultaneous evaluation of human plasma concentration of mycophenolic acid (MPA) and its major metabolites both total and free form. All analytes were extracted from plasma by simple protein precipitation procedure with methanol. Samples for determination of their free form concentration require a preanalytic spin through an ultrafiltration system. The chromatographic separation was completed using C_{18} column at 0.3 ml/min with a gradient condition. Method validation was performed as the United State Food and Drug Administration guidelines for bio-analytical methods concerning precision, accuracy, linearity, selectivity, recovery, and matrix effect. Linearity was obtained over concentration of 0.05–4, 0.5–60, and 0.025–3 μg/ml for total MPA, mycophenolic acid glucuronide (MPAG) and mycophenolic acid acyl-glucuronide (AcMPAG), respectively. The linearity of the method for free form of analytes was confirmed in the range of 10–500, 125–10,000, and 0.5–300 ng/ml for MPA, MPAG, and AcMPAG, respectively. The intra- and interday accuracy ranged from 85.73%–102.01% for total form, and 87.23%–111.89% for free form, and the precisions of all analytes were lower than 15%. The mean recoveries of the analytes ranged from 85.54% to 94.76% and the matrix factor ranged from 0.88–1.06. The developed method is rapid, sensitive and convenient for pharmacokinetic study or therapeutic drug monitoring in patients after oral administration of enteric-coated mycophenolate sodium or mycophenolate mofetil.

Key words: AcMPAG, mycophenolic acid acyl-glucuronide (AcMPAG), mycophenolic acid, ultra-performance liquid chromatography mass spectrometry/mass spectrometry

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INTRODUCTION

Mycophenolate mofetil (MMF) and enteric-coated mycophenolate sodium (EC-MPS) are immunosuppressive drugs prescribed to prevent graft rejection in patients after organ transplantation.[1,2] After oral administration, MMF and EC-MPS are absorbed and primarily hydrolyzed to mycophenolic acid (MPA) in gut. MPA is further metabolized to produce inactive mycophenolic acid glucuronide (MPAG) and active mycophenolic acid acyl-glucuronide (AcMPAG)[1] in liver, gastrointestinal tract, and kidneys by uridine diphosphate glucuronosyltransferase. Additionally, AcMPAG is significant lead to gastrointestinal tract toxicity.[1] Only the free MPA and AcMPAG exert pharmacological effect. Nowadays, total MPA level is used to estimate free MPA level but this assessment is not accurate if interpatient variability is significant, especially in patients with renal failure and hypoalbuminemia. Previous evidence has shown that the incidence of acute graft-versus-host disease associated with low level of free MPA in allograft recipients was increased.[3] As a result, it is important to measure free MPA and its metabolite concentrations for accurate treatment of individual transplant patients. Several published methods, e.g., liquid chromatography (LC) ultraviolet, LC-mass spectrometry (LC-MS)/MS, and immunoassay, have been performed to evaluate plasma concentrations of total MPA, MPAG, and AcMPAG,[4-11] but none of them consist of triple analytes quantification for total or free MPA, MPAG, and AcMPAG. Thus, an UPLC–MS/MS method was validated for the simultaneous quantification of free and total forms of MPA and its metabolites in human plasma.

MATERIALS AND METHODS

Chemicals and materials

MPA-d3, MPA acyl-beta-D-glucuronide and MPA beta-D-glucuronide were obtained from Santa Cruz Biotechnology Inc. (USA). MPA was purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetic acid, formic acid and ammonium acetate were provided by Merck (Germany). LC-MS grade methanol was purchased from Duksan (Duksan, Korea). Deionized water was prepared from distilled water using Labconco (MO, USA). Blank human plasma was derived from healthy volunteers and stored at −20°C until used. An unknown sample of a kidney transplant patient medicated with 540 mg twice daily of EC-MPS was also determined to approve method competency.

Ethical approval

The study protocol was approved by the Institutional Review Board (IRB) of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB no. 463/61) and was registered in Thai Clinical Trials Registry (TCTR20190326003).

Ultra-performance liquid chromatography and tandem mass spectrometry conditions

All measurements were performed using an LCMS-8060 system (Shimadzu, Japan). The detector was used positive electrospray ionization interface and multiple reaction monitoring (MRM) mode for quantitative analysis. The separation of all analytes was carried on Luna® C_{18} (100 mm × 2 mm, 3 μm) column. The temperature of autosampler and column was reserved at 4°C and 40°C, respectively. The mobile phase A (5% methanol in water, v/v) and mobile phase B (methanol containing 2 mM ammonium acetate with 0.1% formic acid) were delivered at 0.3 ml/min with a 10 min total run time. The gradient program of mobile phase was: 30% B >90% B (0–2.5 min), 90% B (2.5–7.5 min), 30% B (7.5–10 min). MRM transitions of MPA, MPAG, and AcMPAG, and MPA-d3 were m/z 321.25 >207.10, 514.00 >321.15, 514.45 >321.20 and 324.20 >210.10. Heating gas: 10 L/min; nebulizing gas: 3 L/min; drying gas: 10 L/min; probe voltage: 4.0 kV. Interface, desolvation line, and heat block temperature was 300°C, 250°C, and 400°C, respectively.

Preparation of standard solutions

MPA, MPAG, AcMPAG, and MPA-d3 stock solutions were prepared in methanol with final concentrations of 1, 1.25, 0.5, and 1 mg/ml, respectively, and were preserved at −20°C until use. The calibration curves in human plasma were prepared between 0.05 and 4 μg/ml range for total MPA, 0.5–60 μg/ml for total MPAG and 0.025–3 μg/ml for total AcMPAG; 10–500 ng/ml for free MPA, 125–10,000 ng/ml for free MPAG and 5–300 ng/ml for free AcMPAG. In all cases, three QC samples (low quality control [LQC], middle quality control [MQC] and high quality control [HQC]) were used and prepared with a final concentration shown in Table 1.

Preparation of calibrators, quality control samples, and patient samples

For total form analysis, 90 μl aliquot of blank plasma was spiked with 10 μl of IS (5,000 ng/ml MPA-d3) working solution and 10 μl of mixed standard solution to prepare calibrators and QC samples, while patient plasma (100 μl) was spiked with 10 μl of IS. Plasma proteins in all samples were precipitated by addition of 890 μl methanol followed by vigorous mixing and centrifugation at 12,000 rpm, 4°C, within 10 min. Supernatant was filtered pass through a 0.22 Nylon filter (ANPEL Laboratory Technologies, China). The filtrate was diluted with methanol in a 1:2 proportion and 2 μl aliquot used for analysis.

For free form analysis, 500 μl aliquot of blank or patient plasma was filtered through an Amicon's Centrifree (Millipore Corp., Bedford, MA) by centrifugation at 14,000 g, 4°C for 30 min. A 90 μl aliquot of ultrafiltrate from blank plasma was spiked with 10 μl of IS (500 ng/ml) and 10 μl of mixed standard solution, while ultrafiltrate from patient

Table 1: Intra- and interday accuracy and precision (*n*=5)

levels	Concentration			Intraday accuracy (%)/ precision (% CV)			Interday accuracy (%)/ precision (% CV)		
	MPA	MPAG	AcMPAG	MPA	MPAG	AcMPAG	MPA	MPAG	AcMPAG
Total (μg/ml)					*n*=5			*n*=15	
LLOQ	0.05	0.5	0.025	104.80/2.89	95.04/2.77	92.80/7.08	103.60/1.16	97.52/2.23	100.80/7.94
LQC	0.15	1.5	0.075	85.73/2.31	90.76/5.86	90.93/4.45	86.09/1.29	90.72/4.11	98.49/6.71
MQC	1.5	30	1	93.73/3.61	89.33/4.86	92.34/5.78	97.21/3.13	91.00/5.35	99.65/7.80
HQC	3	50	2	88.18/0.99	94.22/4.05	102.01/2.91	95.84/7.06	96.85/5.71	101.40/2.90
Free		ng/ml			*n*=5			*n*=15	
LLOQ	10	125	5	102.01/2.26	101.38/1.32	95.42/13.18	103.77/3.29	101.36/2.97	104.33/2.81
LQC	30	350	15	96.36/1.75	97.55/2.02	87.23/7.09	97.78/1.74	98.49/2.24	95.02/7.17
MQC	200	4000	100	108.99/2.68	90.53/4.00	98.38/4.75	111.89/2.62	93.37/4.17	104.26/5.14
HQC	400	8000	200	108.79/2.23	91.96/1.84	89.35/3.79	108.76/0.45	93.33/1.64	93.53/4.45

MPA: Mycophenolic acid, CV: Coefficient of variation, LQC: Low quality control, MQC: Middle quality control, HQC: High quality control, MPAG: Mycophenolic acid glucuronide, AcMPAG: Mycophenolic acid acyl-glucuronide, LLOQ: Lower limit of quantification

plasma (100 μl) was spiked with 10 μl of IS. Methanol (590 μl) was added to each sample. After mixing and centrifugation, the supernatant was injected 2 μl.

Method validation procedures

Blank plasma derived from six difference drug-free healthy volunteers were spiked with the analytes at the lowest concentration of standard curve (lower limit of quantification [LLOQ]) and then used to determine selectivity of method. The peak area of interferences should be lower than 20% than peak area of LLOQ solution. Matrix effect was measured at LQC and HQC samples by comparing the peak areas of analytes and IS from blank plasma which spiked with analytes after extraction to those of the analytes and IS from neat solution at equivalent concentration and reported as matrix factor. The matrix factor of each sample should be in the range of 0.8–1.2. The sensitivity test was determined by injection of five replicates of LLOQ samples in three separate validation batches. The accuracy and precision of each LLOQ sample should be lower than 20%.

Six points standard calibration curves were constructed by plotting peak area ratios of each analyte (peak area of analyte/peak area of IS) against nominal analyte concentrations. A $1/x^2$ (x: Concentration) weighting factor was used to derive the slope, intercept and correlation coefficient. The coefficient of determination (r^2) of the calibration curves for all analytes should be ≥ 0.99.

Batches of five replicates of LLOQ and QC samples (LQC, MQC, and HQC) from freshly prepared calibration curves on the same day were determined for intraday precision and accuracy of method. To evaluate interday accuracy and precision, all levels of QC samples were analyzed on three separate validation days. The acceptance criteria for interday accuracy which expressed as %bias should be within ± 15% for LQC, MQC, and HQC except for the LLOQ that can be within ± 20% of the nominal concentration. Similar to acceptance criteria for interday precision, the results were should be ≤15%.

Extraction recovery of all analytes was investigated in replicate QC samples (n = 5) by comparing the mean area response of each analyte in pre-extracted QC samples to that of post-extracted QC samples which contained analytes at various concentration of the analytes.

RESULTS

Quantification of MPA, MPAG, and AcMPAG

Run-time analysis of MPA, MPAG, and AcMPAG was 10.0 min per sample that giving retention times of 3.5, 3.9, and 4.2 min, respectively. MPA-d3 co-elute with MPA and was used as IS for all analytes. Representative MRM chromatograms for each analyte in spiked blank plasma and ultrafiltrate are shown in Figures 1-3.

Method validation

Selectivity and matrix effect

There was no peak area of endogenous molecule interference with the peak area of all analytes in blank plasma and ultrafiltrate compared to LLOQ samples [Figure 1]. The average matrix factor values for total and free form of analytes were ranged from 0.93–1.03 to 0.88–1.06, respectively.

Accuracy and precision

Accuracy and precision results for total and free analytes are summarized in Table 1. All results are within acceptance criteria recommended by Food and Drug Administration guidance.

Linearity and sensitivity

The coefficient of determination (r^2) values were ≥0.99 for all calibration curves. LLOQ values were 0.05 μg/ml (total MPA), 0.5 μg/ml (total MPAG), 0.025 μg/ml (total AcMPAG), 10 ng/ml (free MPA), 125 ng/ml (free

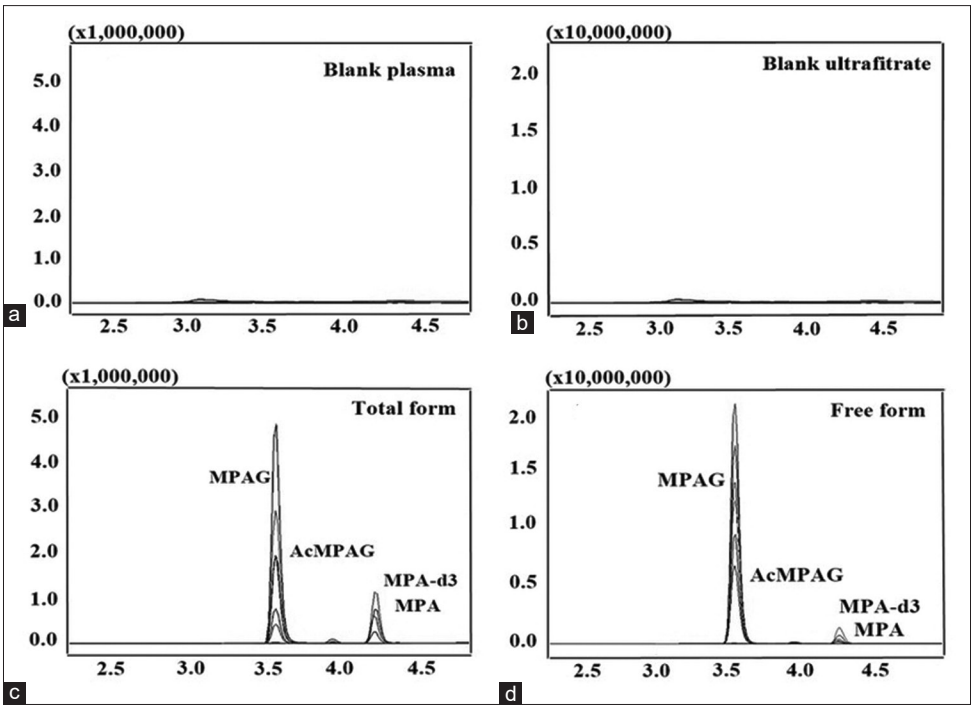

Figure 1: Chromatograms: Blank plasma (a), ultrafiltrate (b), plasma sample (c and d)

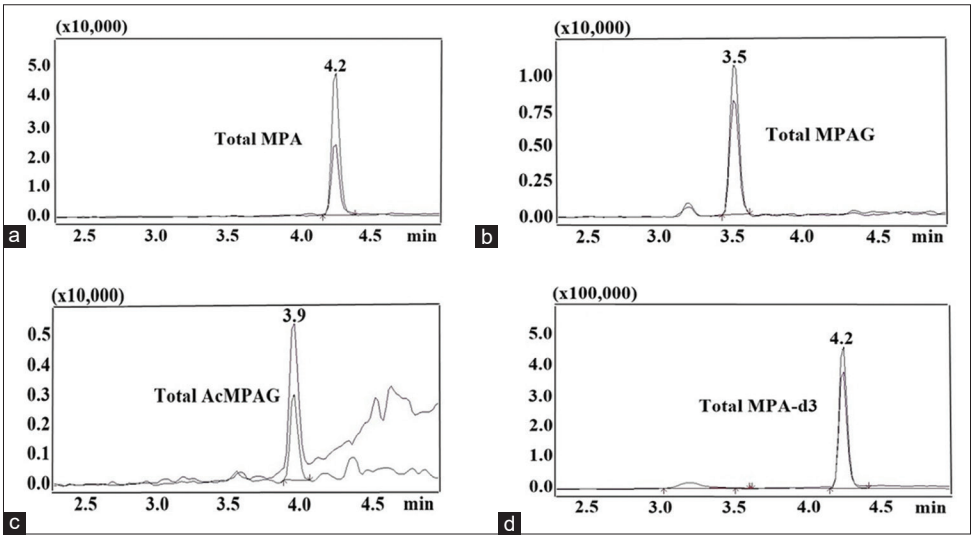

Figure 2: Chromatograms: Blank plasma spiked at lower limit of quantification level of the analytes

MPAG), and 5 ng/ml (free AcMPAG), as shown in Table 1. A chromatogram of blank sample and chromatograms with analyte concentrations at LLOQ are shown in Figures 2 and 3.

Recovery

The extraction recoveries from human plasma, the overall mean recovery values of total MPA, MPAG, and AcMPAG were 91.5%, 86.58%, and 87%, respectively. Recovery efficiency results for free analytes were MPA_92.55%, MPAG_90.72%, and AcMPAG_90.06% [Table 2].

The method showed good quantification of the analytes in a kidney transplant patient plasma as shown in Figure 1.

DISCUSSIONS

We developed a simple one-step sample pretreatment method including plasma protein-precipitating procedure for total form analysis and ultrafiltration procedure for free form analysis with a single internal standard, MPA-d3. Methanol was chosen as the precipitation solvent because it exhibited better effect than acetonitrile and provided acceptable recovery for the analytes and IS. For analysis

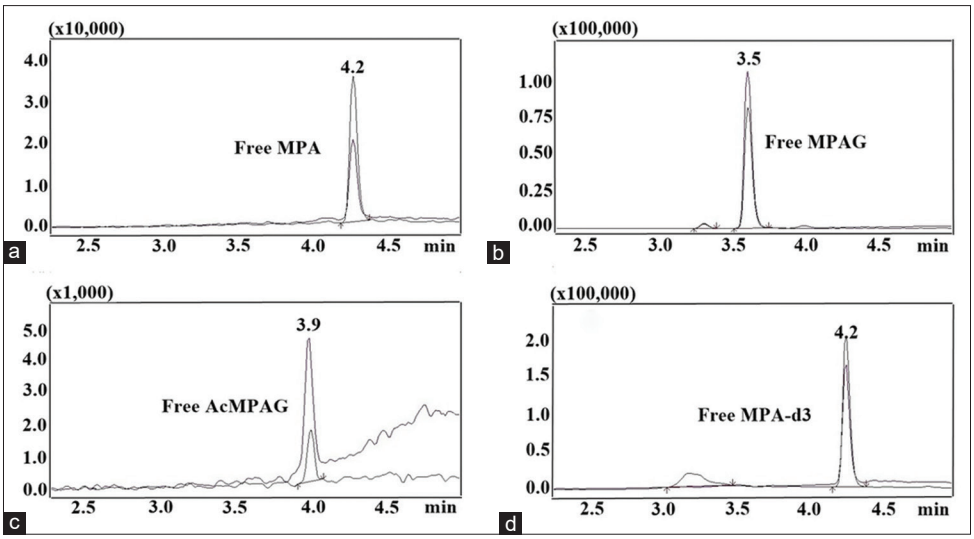

Figure 3: Chromatograms: Ultrafiltrate spiked at lower limit of quantification level of the analytes

Table 2: Matrix effect and recovery of total and free form of analytes (*n*=5)

Compound	Quality control	Matrix factor (mean±SD, % CV)	Percentage recovery (percentage recovery, % CV)
Total MPA	LQC	1.00±0.04 (3.68)	92.12 (5.65)
	MQC	-	88.21 (4.87)
	HQC	0.97±0.126 (13.03)	94.18 (5.07)
Total MPAG	LQC	1.03±0.033 (3.20)	87.00 (3.26)
	MQC	-	85.73 (5.81)
	HQC	0.97±0.101 (10.42)	87.02 (4.94)
Total AcMPAG	LQC	1.02±0.334 (3.31)	89.33 (7.79)
	MQC	-	85.54 (8.66)
	HQC	0.93±0.090 (9.59)	86.12 (6.66)
Free MPA	LQC	1.06±0.04 (4.07)	90.85 (3.78)
	MQC	-	94.76 (2.29)
	HQC	0.92±0.03 (3.24)	92.04 (1.66)
Free MPAG	LQC	1.05±0.04 (4.21)	90.58 (2.23)
	MQC	-	91.82 (2.37)
	HQC	0.92±0.02 (2.42)	89.77 (2.29)
Free AcMPAG	LQC	1.00±0.04 (4.42)	88.07 (4.59)
	MQC	-	91.90 (2.47)
	HQC	0.88 ± 0.03 (3.16)	90.20 (2.63)

LQC: Low quality control, MQC: Middle quality control, HQC: High quality control, MPA: Mycophenolic acid, SD: Standard deviation, CV: Coefficient of variation, MPAG: Mycophenolic acid glucuronide, AcMPAG: Mycophenolic acid acyl-glucuronide

of the free form, ultrafiltration technique was chosen over equilibrium dialysis because the process could be completed within 30 min compared to several hours required for the latter procedure.

The assay consisted of a few logical steps that can be easily performed in any laboratory and showed to be free of matrix effect for all analytes [Table 2]. The mobile phase contained formic acid and ammonium acetate to produce symmetrical peak and good resolution which lead to raise the sensitivity of the method. The gradient program was adapted from that of Delavenne *et al*.[6] and Kawanishi *et al*.,[7] in which free and total forms of MPA, MPAG, and AcMPAG, except the free form of the latter were analyzed. AcMPAG is significantly degraded after five days[4] and is sensitive to degradation during blood sampling, handling and storage.[12] Various factors such as type of anticoagulant, temperature and pH have been reported to affect its stability.[13] In the present study, we added 10% acetic acid into plasma samples in the ratio of 20 µl per 1 ml plasma to increase the stability of AcMPAG as described by Kawanishi *et al*.[7] The triple quadrupole mass spectrometer used showed good mass spectrum response values in the positive ion modes for simultaneous detection and did not demonstrate any significant ion suppression or enhancement. A single internal standard, MPA-d3, was employed.

This method was successfully validated. Calibration curves were generated at six levels of the concentration range for total and free form of MPA, MPAG, and AcMPAG which were appropriate to determine patient samples. There was a very good correlation between concentration and response. The intra- and interday precision and accuracy were considered acceptable range, demonstrating that results are reproducible. LLOQ of this study shows higher sensitivity than others studies.[5] Percentage extraction recoveries of the analytes from ultrafiltrate and plasma was >85%, indicating an acceptable reliability. The method exhibited no significant matrix effect as all matrix factors of all analytes were within acceptable limit.

CONCLUSION

A reliable UPLC–MS/MS method to quantify both total and free form of MPA, MPAG, and AcMPAG in human plasma and ultrafiltrate has been validated. This method was profitably applied to determine blood levels and assess the pharmacokinetic profiles of all analytes in patients receiving MMF or EC-MPS.

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Conflicts of interest

There are no conflicts of interest.

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