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Sensitive and Validated LC-MS/MS Methods to Evaluate Mycophenolic Acid Pharmacokinetics and Pharmacodynamics in Hematopoietic Stem Cell Transplant Patients

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ABSTRACT:

Monitoring of pharmacodynamics in addition to pharmacokinetics is one of strategies to

individualize mycophenolate mofetil (MMF) therapy. The purpose of this study was to

develop sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods

for evaluation of the pharmacokinetics and pharmacodynamics of mycophenolic acid (MPA).

Concentrations of mycophenolic acid glucuronide (MPAG), mycophenolic acid

acyl-glucuronide (AcMPAG), as well as unbound MPA and MPAG, were determined, and

inosine-5′-monophosphate dehydrogenase (IMPDH) activity was calculated by measuring

concentrations of produced xanthosine-5′-monophosphate (XMP) and intracellular

adenosine-5′-monophosphate (AMP) after incubation of peripheral blood mononuclear cell

(PBMC) lysates. A metal-free Mastro^{TM} column and 2 gradient patterns were used to improve

the quantification limit of XMP to 0.1 µM. In the clinical MPA concentration range, the

linearity of the calibration curve, inter- and intra-day precision, and accuracy satisfied the

relevant FDA guidelines. The MPA concentrations in hematopoietic stem cell transplant

(HSCT) patients determined by the enzyme assay and the present LC-MS/MS method

showed a good correlation ($r^2 = 0.95$, $p < 0.001$). In this study, we report sensitive and

validated LC-MS/MS methods to evaluate the pharmacokinetics and pharmacodynamics of

3

MPA, which are sufficiently sensitive to assess small quantities of PBMC lysates collected

shortly after HSCT.

Key words: mycophenolic acid, IMPDH, LC-MS/MS, therapeutic drug monitoring

INTRODUCTION

Mycophenolate mofetil (MMF) is an immunosuppressive agent used worldwide for the

prevention of rejection after solid-organ transplantations (Kaufman *et al*., 2004), and it is also

used in hematopoietic stem cell transplant (HSCT) recipients for graft-*versus*-host disease

(GVHD) prophylaxis (Minagawa *et al*., 2012). The therapeutic effect of mycophenolic acid

(MPA), the active and hydrolyzed form of MMF, is based on the potent, selective, and

reversible inhibition of inosine-5′-monophosphate dehydrogenase (IMPDH). IMPDH is the

rate-limiting enzyme involved in *de novo* synthesis of guanosine nucleotides; IMPDH

catalyzes the oxidation of inosine-5′-monophosphate (IMP) to xanthosine-5′-monophosphate

(XMP) by a nicotinamide adenine dinucleotide (NAD^+)-dependent reaction (Allison *et al*.,

2000). Because this XMP synthesis pathway is essential for the mitogenic function of

lymphocytes, the inhibitory effect of MPA on IMPDH stops T cell proliferation and thereby

leads to immunosuppression (Allison *et al*., 2000).

MPA is glucuronidated in the liver mainly to an inactive metabolite, mycophenolic acid

glucuronide (MPAG), and partially to a pharmacologically active metabolite, mycophenolic

acid acyl-glucuronide (AcMPAG) (Shipkova *et al*., 2003). Fig. 1 shows the structure of each

compound. MPAG undergoes enterohepatic recirculation (Bullingham *et al*., 1998). MPA is

highly bound to albumin and the unbound fraction of MPA is approximately 1−3% of the total concentration (Nowak *et al*., 1995). MMF has large inter-individual variability in pharmacokinetics and requires therapeutic drug monitoring (TDM) (Staatz *et al*., 2007). While the recommended target range for the MPA area under the curve from 0 to 12 hours (AUC_{0-12}) in renal transplant recipients is 30–60 mg · h/L (Shaw *et al*., 2001), the optimal MMF dose for GVHD prophylaxis in HSCT patients has not been established (Minagawa *et al*., 2012). Moreover, it was reported that pre-transplant IMPDH activity is associated with clinical outcome in renal transplant patients, which suggests that IMPDH activity should be investigated as a suitable marker in the field of transplantation (Glander *et al*., 2004). Recently, it was reported that the area under the effect curve (AUEC) of IMPDH activity on day 21 after HSCT was associated with non-relapse mortality and overall mortality, but was not associated with GVHD (Li *et al*., 2014). Further studies are needed on the pharmacokinetics and pharmacodynamics of MPA in HSCT patients.

To evaluate IMPDH activity in peripheral blood mononuclear cells (PBMCs), various methods such as liquid chromatography-tandem mass spectrometry (LC-MS/MS) have been described (Glander *et al*., 2009; Maiguma *et al*., 2010; Laverdière *et al*., 2012). However, there remain several limitations in established IMPDH assay methods: calibration standards

are prepared by dilution without PBMC matrix (Glander *et al.*, 2009; Laverdière *et al.*, 2012),

and the quantification limit of XMP is 0.25 µM, which is insufficient to determine the

concentrations of XMP and AMP in samples obtained from HSCT patients.

Therefore, we developed sensitive LC-MS/MS methods to measure plasma

concentrations of MPA, its 2 metabolites, and unbound MPA and MPAG, as well as IMPDH

activity in PBMC lysates. We used PBMC matrix as a calibration standard for our

LC-MS/MS analyses according to the Bioanalytical Method Validation (BMV) guideline

(FDA, 2013), and aimed to improve the sensitivity of XMP by measurements using a specific

column and 2 gradient patterns. We evaluated and validated the presently reported

LC-MS/MS methods in samples from 3 healthy volunteers and 6 HSCT patients.

EXPERIMENTAL

Chemicals and reagents

MPA was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). MPAG was

purchased from Analytical Services International Ltd. (London, UK). AcMPAG and XMP

disodium salt were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Mycophenolic acid-d3 (MPA-D$_3$) was purchased from Toronto Research Chemicals Inc.

(North York, Canada). Adenosine-5′-monophosphate (AMP) sodium salt from yeast, IMP

disodium salt from yeast, and NAD^+ were purchased from Nacalai Tesque Inc. (Kyoto,

Japan). 8-Bromo-adenosine-5′-monophosphate (Br-AMP) sodium salt was purchased from

Jena Bioscience (Jena, Germany). All the chemicals used were of the highest grade available.

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

For analysis of MPA, its metabolites, and unbound MPA and MPAG, stock solutions were

prepared by dissolving MPA (1 mg/mL), MPAG (10 mg/mL), AcMPAG (1 mg/mL) and

MPA-D_3 (1 mg/mL) in methanol. All solutions were stored at -80 °C. Working solutions were

prepared by diluting stock solutions with methanol to the appropriate concentrations (200

µg/mL MPA, 1600 µg/mL MPAG, and 40 µg/mL AcMPAG) for analysis of MPA and its

metabolites, and further diluting solutions to 0.5 µg/mL MPA and 50 µg/mL MPAG for

analysis of unbound MPA and MPAG. All diluted working solutions were stored at -20 °C.

Calibration standards were prepared from the working solutions to yield target concentrations

of MPA (0.1 to 20 µg/mL), MPAG (0.8 to 160 µg/mL), and AcMPAG (0.02 to 4 µg/mL) by

diluting solutions with acidified plasma for analysis of MPA and its metabolites. For analysis

of unbound MPA and MPAG, MPA and MPAG calibration solutions were prepared at

concentrations ranging from 5 to 200 ng/mL and 500 to 20000 ng/mL, respectively, using

purified water. The working solution of internal standard MPA-D$_3$ was prepared from stock

solution at the time of the assay by dilution with methanol. Quality control (QC) samples of

MPA, MPAG, and AcMPAG were prepared at low, medium, and high concentrations in

acidified plasma of healthy subjects and kept at -20 °C.

For analysis of IMPDH activity, XMP (1 mM) and Br-AMP (1 mM) stock solutions

were prepared in water, and AMP (1 mM) stock solution was prepared in 0.5% ammonium

hydroxide. All solutions were stored at -80 C°. Working solutions of 100 µM XMP and 500

µM AMP were stored at -20 °C. Calibration standards were prepared for XMP (0.1 to 10 µM)

and AMP (0.5 to 50 µM) by dilution with a mixture of the supernatant from the PBMC lysate,

incubation buffer, and stop solution. The working solution for internal standard Br-AMP was

prepared from stock solution at the time of assay by dilution with 95% water and 5%

methanol.

Clinical sample collection

Blood samples were collected in 3 mL heparinized collection tubes from 6 HSCT patients

before and 1, 2, 4, and 8 hours after MMF administration on day 7 and 21 after

transplantation. Blood samples were centrifuged at 15000 g for 10 minutes, and 20 µL of 10% acetic acid was added to 1 mL of the separated plasma sample for stabilization of AcMPAG. Unbound MPA was separated by ultrafiltration of 500 µL plasma using Amicon Ultra 30 K centrifugal filter devices (Merck Millipore Ltd., Carrigtwohill, Ireland) at 14000 g for 10 min. MPA concentrations of all clinical samples were also determined using an enzyme assay (Roche total MPA assay on Cobas 6000; Roche Diagnostics, Tokyo, Japan), and the results of this enzyme assay were compared to those obtained using the LC-MS/MS method described herein.

PBMCs were isolated from the remaining blood sample by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), and frozen at -20 °C until analysis. Area under the plasma concentration time curve from 0 to 8 hours (AUC_{0-8}) of MPA and its metabolites was determined using the linear trapezoidal method. This clinical study was performed in accordance with the Declaration of Helsinki and its amendments and was approved by the Kyoto University Graduate School and Kyoto University Hospital Ethics Committee. Written informed consent was obtained from each patient.

Assay condition

IMPDH activity was evaluated in PBMC lysates based on previous reports (Glander *et al*., 2009; Maiguma *et al*., 2010; Laverdière *et al*., 2012) with slight modifications. First, 300 µL of water was added to a sample of thawed PBMC lysate, after which the mixture was agitated by vortexing to break down cell membranes and centrifuged at 1000 *g* for 2 min. The reaction was initiated by adding 50 µL of the supernatant to 52 µL of incubation mixture consisting of 1.6 mM phosphate buffer (consisting of sodium dihydrogen phosphate and sodium monohydrogen phosphate, pH 7.4), 3.9 mM potassium chloride, 1.2 mM of IMP, and 0.6 mM of NAD^+ (final concentrations). The incubation assay was performed at 37 °C for 150 min. The reaction was stopped by the addition of 5 µL of 2.5 M perchloric acid followed by 10 µL of 3 M potassium monohydrogen phosphate. The mixture was centrifuged for 5 min and the supernatant was used for determination. AMP concentrations were determined using another sample without the addition of IMP and NAD^+. IMPDH activity was calculated based on XMP formation and normalized to intracellular AMP using the following equation, in which produced XMP (*$XMP_{produced}$*) and AMP (*$AMP_{measured}$*) concentrations are expressed in micromoles per liter and incubation time (*t_i*) is expressed in seconds:

IMPDH activity ($\mu mol \cdot s^{-1} \cdot mol^{-1}$ AMP) = $XMP_{produced} \cdot 10^6 / (t_i \cdot AMP_{measured})$.

11

Internal standards for LC-MS/MS measurements

MPA-D_3 and Br-AMP were chosen as internal standards for LC-MS/MS assays (Delavenne *et al*., 2011; Laverdière *et al*., 2012). Fifty µL of acidified plasma sample was mixed with 100 µL of 0.2 µg/mL MPA-D_3 internal standard for analysis of MPA and its metabolites, 50 µL of ultra-filtrated plasma sample was mixed with 10 µL of 0.2 µg/mL MPA-D_3 internal standard for analysis of unbound MPA. On one hand, 60 µL of incubation mixture was mixed with 10 µL of 100 µM Br-AMP internal standard for analysis of IMPDH activity. Each sample was filtered through a Cosmonice Filter W (Nacalai Tesque Inc., Kyoto, Japan) with a pore size of 0.45 µm for LC-MS/MS determination.

LC-MS/MS conditions

The LC-MS/MS system (LCMS-8040; Shimadzu, Kyoto, Japan) was run in multiple reactions monitoring mode with positive ion mode for determinations. The dwell time was 0.1 sec for all compounds. The columns were kept at 40 °C. The autosampler was kept at 4 °C, and 1 µL sample solution was injected for each determination. LabSolutions LCMS software (Shimadzu) was used to control the instruments and to process the data.

For analysis of MPA, its metabolites and unbound MPA and MPAG, chromatographic separation was achieved with a Gemini C18 column (150 mm × 2.00 mm, particle size 5 µm; Phenomenex, Torrance, CA, USA). The column was eluted with combinations of water (A) and methanol (B) containing 0.1% formic acid and 2 mM ammonium acetate, respectively. The flow rate was 0.2 mL/min. The elution gradient was: 0–5 min, linear from 30 to 90% B; 5–9 min, 90% B; 9–12 min, 100% B; 12–15 min, 30% B. The retention times were as follows: MPA, 6.92 min; MPAG, 5.86 min; AcMPAG, 6.78 min; MPA-D_3, 6.92 min. The molecules were detected according to the following mass transitions: 321.1 > 207.1 (MPA), 514.1 > 207.1 (MPAG), 514.2 > 207.1 (AcMPAG), and 324.2 > 210.0 (MPA-D_3).

For analysis of IMPDH activity, a metal-free Mastro^{TM} column (150 mm × 2.1 mm, particle size 3 µm; Shimadzu GLC, Tokyo, Japan) was used, and the solvents were water (A) and methanol (B) containing 1 mM ammonium acetate, respectively. The flow rate was 0.2 mL/min, and we used 2 elution gradient patterns. The gradient for XMP analysis was: 0–4.5 min, linear from 10 to 50% B; 4.5–5.0 min, 100% B; 5.0–5.5 min, linear from 100 to 10% B; 5.5–10.0 min, 10% B. The gradient for AMP analysis was: 0–4.5 min, linear from 0 to 50% B; 4.5–5.0 min, 100% B; 5.0–5.5 min, linear from 100 to 0% B; 5.5–10.0 min, 0% B. Retention times in the XMP assay were 1.64 min for XMP and 3.60 min for Br-AMP, and

retention times in the AMP assay were 4.11 min for AMP and 4.83 min for Br-AMP. The

molecules were detected according to the following mass transitions: 365.1 > 97.2 (XMP),

348.1 > 136.2 (AMP), and 428.0 > 216.0 (Br-AMP).

Method validation

The intra- and inter-day precision levels were assessed based on the coefficient of variation

(CV, %), whereas the accuracy (%) was determined by the following equation:

Accuracy (%) = (QC$_{measured}$/QC$_{reference}$) × 100

where $QC_{measured}$ is the measured QC concentration and $QC_{reference}$ is the reference QC

concentration. Assessments were validated by analyzing 5 replicates of QC samples. The

stability of MPA and its metabolites in acidified plasma samples from patients was examined

at 4 °C continuously for the first 24 hours after blood sampling. XMP and AMP stability in

PBMC lysate samples from healthy subjects was examined at -20 °C continuously for 10

weeks after PBMC extraction. Seven- or 8-point calibration curves were prepared by adding

standard products to blank matrix. The coefficient of determination (r^2) was used to evaluate

the linearity of the calibration curve. Matrix effects were determined by comparing the peak

areas (corrected by internal standards) of compounds added to acidified plasma or PBMC

14

matrix with those added to water.

The internal levels of XMP or AMP in the reagents of IMP and NAD$^+$ or PBMC samples were determined by analyzing incubation samples with or without them. The produced XMP concentrations at 0, 60, 120, and 150 min after incubation were determined using PBMCs from 3 healthy subjects. The concentrations of AMP and protein in PBMC lysate samples of various concentrations from 3 healthy subjects were determined. Protein concentrations were measured by the Bradford method.

Inhibition assay of MPA and AcMPAG

MPA-mediated inhibition of IMPDH activity was assessed in PBMC lysate samples from 3 healthy subjects. MPA and AcMPAG were added to each PBMC lysate sample to a final concentration ranging from 0 to 300 ng/mL and from 0 to 3000 ng/mL, respectively, and incubated as previously described. MPA- or AcMPAG-mediated inhibition of IMPDH activity was assayed by measuring residual IMPDH activity after the addition of MPA or AcMPAG. Pharmacodynamic parameters were calculated by the following sigmoid inhibitory maximum effect model:

$$E = E_0 \cdot [1 - I_{max} \cdot C_p^{\gamma}/(IC_{50}^{\gamma} + C_p^{\gamma})]$$

where E_0 is baseline IMPDH activity, I_{max} is maximal IMPDH inhibition, C_p is the MPA

plasma concentration, IC_{50} is the MPA concentration that causes 50% of maximal IMPDH

inhibition, and γ is the Hill coefficient that governs the slope of the MPA concentration *versus*

IMPDH activity. The E_0 values were taken as the original IMPDH activity of each subject

without the addition of MPA and AcMPAG.

RESULTS

Method validation

We initially confirmed the selectivity of the chromatographic method for the targeted analytes

and the internal standard. Fig. 2 shows the chromatograms of every tested analytes in

acidified plasma matrix (A–D) and in PBMC matrix (E and F). As shown in Table 1, the

calibration curve was linear in the examined range. Matrix effects of each compound and

each concentration were stable (Table 1). Table 2 shows the inter- and intra-day precision

(CV, %) and accuracy (%). All CVs were less than 15% and accuracy ranged from 85% to

115%, with the exception of the lower limit of quantification for XMP (in this case, CVs

were less than 20% and accuracy ranged from 80% to 120%). MPA, MPAG, and AcMPAG

were stable at 4 °C under acidic conditions until 24 hours after blood sampling (101.9 ± 4.6%

16

of initial values, mean ± SD, n = 3). IMPDH activity in PBMC lysate samples stored at

-20 °C was stable until 10 weeks after extraction (105.8 ± 14.2% of initial values, mean ± SD,

n = 3).

We further measured potential contamination by XMP and AMP in our commercial

sources of IMP and NAD^+ as previously suggested (Laverdière *et al.*, 2012). As shown in Fig.

3, the IMP and NAD^+ used for the incubation medium contained 0.1 μM XMP and 6.1 μM

AMP, respectively. The combination of 1.2 mM IMP and 0.6 mM NAD^+ in the incubation

medium led to linear formation of XMP in 3 healthy subjects under the assay conditions

described above (Fig. 4A). The AMP and protein concentrations in PBMC lysates from 3

healthy subjects showed a good correlation, but a certain amount of protein was contained in

each sample at 0 μM AMP (Fig. 4B). The addition of MPA (0–300 ng/mL) or AcMPAG (0–

3000 ng/mL) to the PBMC lysates showed a similar inhibition curve in 3 healthy subjects

(Fig. 5). IC_{50}, I_{max}, and γ were 1.65 ± 0.92 ng/mL, 0.99 ± 0.03, and 1.08 ± 0.52, respectively,

for MPA, and 9.48 ± 1.52 ng/mL, 0.98 ± 0.01, and 1.00 ± 0.23, respectively, for AcMPAG

(mean ± SD, n = 3 for each result).

Measurement of clinical samples

17

Time course data was obtained from 6 HSCT patients on day 7 and 21. The MPA concentrations determined by the enzyme assay and LC-MS/MS showed a good correlation ($r^2 = 0.95$, $p < 0.0001$, Fig. 6A). The AUC_{0-8} ratio of MPAG/MPA varied from 10 to 40 in each individual, but this ratio was similar across measurements in each patient, and thus showed low intra-individual variability (Fig. 6B). The AUC_{0-8} ratio of AcMPAG/MPA showed high inter- and intra-individual variability (Fig. 6C). Fig. 7 shows a typical time profile of MPA concentration and IMPDH activity on day 7 after oral administration of 30 mg/kg/day, three times per day of MMF.

DISCUSSION

Based on a previous report (Delavenne *et al*., 2011), we developed a method to analyze plasma concentrations of MPA, its metabolites MPAG and AcMPAG, and unbound MPA and MPAG. Analytical methods for IMPDH activity have been published previously (Glander *et al*., 2009; Maiguma *et al*., 2010; Laverdière *et al*., 2012), but there are limitations on these established methods: they did not use PBMC matrix for calibration standards, and the quantification limit of XMP was 0.25 µM, which was not sufficient to determine the amount of XMP shortly after HSCT. The LC-MS/MS method described here used PBMC matrix for

18

calibration standards and allowed the sensitive measurement of IMPDH activity. Our

LC-MS/MS method can determine XMP concentrations as low as 0.1 µM by using a

metal-free column that suppresses the adsorption of phosphate compounds onto the column

surface. Furthermore, the analytical LC-MS/MS methods reported herein were fully validated

with regard to correct peak shapes, linearity within the range of clinical concentrations, and

reproducibility and accuracy of inter- and intra-day measurement (Fig. 2; Tables 1, 2). We

demonstrated that XMP and AMP could be quantified even in the small quantities of PBMC

lysates collected from patients on day 7 and 21 after HSCT.

As stated in a previous report (Laverdière *et al*., 2012), XMP and AMP have been

detected as contaminants in commercially available IMP and NAD^+ (Fig. 3). Therefore, we

determined the amount of XMP in each sample by subtracting the XMP contamination (0.1

µM) from the measured values. AMP concentrations were measured without IMP and NAD^+.

It has been reported that the IC_{50} of MPA for IMPDH activity in human PBMC lysates *in*

vitro is 5−6 nM (1.6−1.9 ng/mL) (Maiguma *et al*., 2010) or 2−3 ng/mL (Glander *et al*., 2001),

but no reports have compared the IC_{50} of MPA to that of AcMPAG. We added 0−300 ng/mL

of MPA or 0−3000 ng/mL of AcMPAG to PBMC lysates from 3 healthy subjects and

incubated them for 150 min; the IC_{50} of MPA (1.65 ± 0.92 ng/mL) coincided with previously

reported values, and the IC$_{50}$ of AcMPAG (9.48 ± 1.52 ng/mL) was about 6 times greater than that of MPA (Fig. 5). IMPDH activity was completely inhibited by 100 ng/mL MPA and 1000 ng/mL AcMPAG. Although small inter-individual variability in IMPDH activity was observed in the 3 healthy subjects, large inter-individual variability in IMPDH activity in HSCT patients is expected, as shown in a previous report (Li *et al*., 2014).

IMPDH activity decreased as the MPA plasma concentration increased, and there was no time delay between the peak MPA concentration and the lowest level of IMPDH activity (Fig. 7). Previous studies show that monitoring pharmacodynamics in addition to pharmacokinetics might be useful for individualized therapy with MPA (Glander *et al*., 2004). Recently, pharmacokinetic and pharmacodynamic analyses were performed in 56 nonmyeloablative HSCT patients (Li *et al*., 2014), but IMPDH activity was determined only on day 21, and the activity on day 7 could not be determined because of myelosuppression resulting from even the nonmyeloablative-conditioning regimen. In addition, determination of IMPDH activity not only at a stable time point but also at an early time point after HSCT is important, because IMPDH activity changes as the chimerism progresses. Utilizing the sensitive analytical method reported herein, it is possible to measure IMPDH activity at time points as early as day 7 after HSCT, which is not possible with previously reported analysis

methods. Therefore, our method should allow for precise evaluations of pharmacokinetics

and pharmacodynamics that will benefit HSCT patients.

Because each sample contains a different number of PBMCs, IMPDH activity must be

corrected by cell number. Although enzyme activity can be normalized using cell count,

protein concentration, and AMP concentration, use of cell count for normalization causes

high intra- and inter-observer variability. We simultaneously determined the amount of AMP

and protein in samples in order to determine the appropriate correction for the number of

PBMCs. Although AMP concentrations and protein concentrations showed a good linear

correlation (Fig. 4B), each sample included a certain amount of excessive protein, which may

have been due to extracellular protein that could not be washed out by the process of PBMC

isolation that was performed on the final samples. It has been reported that the use of multiple

washing steps after cell isolation may remove MPA from cells, leading to an underestimation

of *in vivo* MPA-induced IMPDH inhibition (Glander *et al*., 2012). However, a minimal

washing procedure resulted in residual protein and erythrocyte contamination that may have

influenced the estimation of IMPDH activity when corrected by protein concentration

(Glander *et al*., 2012). Normalization of the results from each sample based on its respective

intracellular AMP concentration minimizes the impact of contaminants on the determination

of IMPDH activity. Therefore, in our study we used a minimal washing procedure and

selected intracellular AMP as the factor by which to correct the number of PBMCs.

The good correlation between MPA plasma concentrations measured using the

LC-MS/MS method reported here and the enzyme assay normally used for routine TDM in

our hospital indicated that our LC-MS/MS method was comparable to the routine automated

method. However, the MPA concentrations measured using the enzymatic method were

overestimated in comparison with those measured using LC-MS/MS, possibly because of the

cross-reaction of AcMPAG in the enzymatic method. Figs. 6B and C show the

inter-individual variability in the AUC ratios of MPAG/MPA and AcMPAG/MPA. Although

the reason for the intra-individual variability in the AcMPAG/MPA ratio is unclear, it might

be caused by differences in the activity of the UDP-glucuronosyl transferases responsible for

metabolism of MPAG and AcMPAG. In future studies, we will investigate the influence of

enterohepatic circulation of MPAG and the effect of AcMPAG on the pharmacodynamics of

MPA using the newly developed LC-MS/MS methods reported here.

CONCLUSION

We developed sensitive and validated LC-MS/MS methods for the analysis of MPA

pharmacokinetics and pharmacodynamics in HSCT patients. Because we needed to improve

the analytical sensitivity of measurements of IMPDH activity for the analysis of samples in

HSCT patients, we used a specific column and 2 gradient patterns, and the quantification

limit of XMP was improved to 0.1 µM, which allowed us to determine XMP in small samples

of PBMCs collected on day 7 after HSCT. The newly developed methods reported here

provide good precision and accuracy according to the BMV guideline of the FDA, and are

convenient for large-scale clinical studies. Precise MPA pharmacokinetic/pharmacodynamic

monitoring will allow clinicians to further optimize MPA-induced immunosuppression for

various diseases and transplantation procedures.

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Figure legends

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

Fig. 2. Chromatograms obtained from acidified plasma matrix containing 6.7 ng MPA (A), 53.3 ng MPAG (B), 1.3 ng AcMPAG (C) and 1.3 ng MPA-D$_3$ (D). Chromatograms obtained from PBMC matrix containing 6.9 pmol XMP and 14 pmol Br-AMP (E) and 34.3 pmol AMP and 14 pmol Br-AMP (F).

Fig. 3. Internal levels of XMP and AMP in a buffer solution only (A), containing IMP and NAD$^+$ (B), containing PBMCs (C), and containing IMP, NAD$^+$, and PBMCs (D). PBMCs were obtained from healthy subjects. Each bar shows the mean $\pm$ SD (n = 3).

Fig. 4. Time profiles of XMP production in 3 healthy subjects (A), and the correlation between AMP and protein concentrations in PBMC lysates from 3 healthy subjects (B). Each symbol represents the mean $\pm$ SD (n = 3) for each subject. XMP production increased linearly with the time of incubation (A: r^2 = 0.89–0.97; p < 0.001). The AMP concentration was strongly correlated with the protein concentration (r^2 = 0.97; p < 0.001).

Fig. 5. *In vitro* inhibition of IMPDH activity by MPA (A) and AcMPAG (B). After PBMC lysates from 3 healthy subjects were incubated for 150 min with several concentrations of MPA or AcMPAG, IMPDH activity was determined. Each figure shows a typical curve from

each subject.

Fig. 6. Correlation of MPA concentrations measured using the enzyme assay with those determined using the LC-MS/MS method (A). AUC_{0-8} ratio of MPAG/MPA (B) and AcMPAG/MPA (C) in 6 patients. Time course data obtained from 6 HSCT patients treated with MMF at the first and third week after transplantation were used in the analyses.

Fig. 7. A typical time course of MPA concentration and IMPDH activity after oral administration of 30 mg/kg/day, three times per day of MMF on day 7 after HSCT.

Table 1. Calibration range, coefficient of determination (r²), and matrix effect

	Calibration range	r^2	Matrix effect ($n = 3$) Mean ± SD (%)		
			Low	Middle	High
MPA	0.1–20 µg/mL	1.00	87.1 ± 3.8	104 ± 5	104 ± 4
MPAG	0.8–160 µg/mL	0.998	100 ± 13	94.6 ± 3.0	97.0 ± 4.6
AcMPAG	0.02–4 µg/mL	1.00	75.0 ± 11.0	94.1 ± 6.7	97.5 ± 3.8
free MPA	5–200 ng/mL	1.00	98.7 ± 4.1	99.7 ± 3.2	101 ± 2
free MPAG	0.5–20 µg/mL	0.999	97.0 ± 4.2	97.8 ± 3.0	96.9 ± 1.9
XMP	0.1–10 µM	0.997	70.3 ± 9.0	76.8 ± 6.4	73.5 ± 4.9
AMP	0.5–50 µM	0.999	90.1 ± 8.9	92.1 ± 2.2	88.4 ± 5.9

Table 2. Intra- and inter-day precision and accuracy values

		CV (%)			Accuracy (%)		
		Low	Middle	High	Low	Middle	High
Intra-day (*n* = 5)	MPA	0.71	1.50	1.93	114	109	101
	MPAG	7.93	5.54	5.37	102	112	112
	AcMPAG	7.04	4.33	7.49	104	110	110
	free MPA	2.84	1.40	0.69	93.8	101	101
	free MPAG	0.61	0.63	0.71	96.5	97.1	104
	XMP	3.24	1.69	1.67	117	93.4	103
	AMP	4.47	1.57	0.72	99.8	106	97.1
Inter-day (*n* = 5)	MPA	5.69	5.06	3.49	97.0	96.1	95.3
	MPAG	4.98	6.24	2.06	93.5	96.3	95.2
	AcMPAG	7.07	5.72	6.12	95.5	90.3	87.3
	free MPA	7.93	3.90	3.02	104	103	98.0
	free MPAG	2.91	4.67	2.96	100	96.0	103
	XMP	19.1	5.89	3.36	114	107	97.6
	AMP	0.29	5.41	3.26	100	99.5	102

Fig. 1

A

B

C

Fig. 2

Fig. 3

	IMP and NAD$^+$ (−)	IMP and NAD$^+$ (+)
PBMCs (−)	**A** — XMP: n.d., AMP: n.d.	**B** — XMP: 0.10, AMP: 6.05
PBMCs (+)	**C** — XMP: n.d., AMP: 2.54	**D** — XMP: 0.13, AMP: 8.96

Fig. 4

Fig. 5

A

IMPDH activity (µmol/s/mol AMP)

20

15

10

5

0

0.1   1   10   100   1000

MPA concentration (ng/mL)

B

IMPDH activity (µmol/s/mol AMP)

20

15

10

5

0

1   10   100   1000   10000

AcMPAG concentration (ng/mL)

Fig. 6

A

Y = 1.18X + 0.72
r^2 = 0.95

MPA concentration by enzyme assay (μg/mL)

MPA concentration by LC-MS/MS (μg/mL)

B

AUC ratio of MPAG / MPA

Pt.1 Pt.2 Pt.3 Pt.4 Pt.5 Pt.6

C

AUC ratio of AcMPAG / MPA

Pt.1 Pt.2 Pt.3 Pt.4 Pt.5 Pt.6

Fig. 7