Quantification of Mycophenolic Acid and Glucuronide Metabolite in Human Serum by HPLC-Tandem Mass Spectrometry

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Background: The potent immunosuppressant mycophenolic acid (MPA) is metabolized to an inactive glucuronide (MPAG). The extent of metabolism varies among individuals, and the MPAG formed can be hydrolyzed to MPA and can displace MPA from serum albumin, creating a potential need to monitor both MPA and MPAG.

Methods: After addition of the carboxybutoxy ether of MPA (MPAC) as internal standard, MPA and MPAG were isolated from serum by acidification followed by solid-phase extraction. Gradient chromatographic separation was performed on a Waters Atlantis reversed-phase liquid chromatography (HPLC) column, and the compounds were quantified by electrospray ionization tandem mass spectrometry (MS/MS) in the multiple-reaction monitoring mode. Results obtained by HPLC-MS/MS were compared with an HPLC assay using ultraviolet detection (HPLC-UV) performed at a reference laboratory.

Results: MPAG, MPA, and MPAC were fully separated during a 7.0-min run time. Precision at both low and high concentrations of MPA and MPAG met the suggested method validation criteria from a consensus panel report on MPA. The extraction efficiencies were 99% for MPA and MPAG. The assay was linear to 16 mg/L for MPA and 200 mg/L for MPAG. Limits of quantification were 0.1 mg/L for MPA and 1 mg/L for MPAG. Regression analysis gave the following results: HPLC-MS/MS = 1.03(HPLC-UV) - 0.03 mg/L ($R^2 = 0.982$) for MPA; and HPLC-MS/MS = 0.93(HPLC-UV) + 0.89 mg/L ($R^2 = 0.967$) for MPAG.

Conclusion: This HPLC-MS/MS assay can be used to reproducibly quantify MPA and MPAG across a large analytical range in serum from organ transplant patients.

Mycophenolic acid (MPA)\(^1\) is the pharmacologically active form of the immunosuppressant prodrug mycophenolate mofetil. MPA is also available as the enteric coated sodium salt. The drug is a reversible noncompetitive inhibitor of inosine monophosphate dehydrogenase type 2, an enzyme that is important in the de novo synthesis of guanosine nucleotides within lymphocytes. Because the mode of action of MPA differs from that of cyclosporine, tacrolimus, and sirolimus, MPA can be co-prescribed as part of a multiple drug regimen.

MPA is predominantly metabolized by glucuronidation to form an inactive metabolite, mycophenolic acid glucuronide (MPAG), as well as a minor acyl glucuronide metabolite. Although inactive, both the pharmacokinetics and concentrations of MPAG have important implications in optimal use of MPA as an immunosuppressant (1). One reason is that serum concentrations of MPAG may be many times higher than those of MPA, particularly in patients with compromised renal function. MPA is ~97% bound to albumin under normal circumstances, but the free (active) fraction of the drug may be increased because of displacement by high circulating concentrations of MPAG (2). In addition, MPAG undergoes enterohepatic recycling, during which time it may be hydrolyzed back to MPA and reabsorbed to produce a secondary peak concentration of the active compound MPA. Moreover, the hepatic conjugating capacity varies among individuals. Some patients may have high metabolic capacity, so that the orally absorbed active MPA is rapidly converted

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\(^{1}\) Nonstandard abbreviations: MPA, mycophenolic acid; MPAG, mycophenolic acid glucuronide; MS/MS, tandem mass spectrometry; SPE, solid-phase extraction; MPAC, mycophenolic acid carboxybutoxy ether; and MRM, multiple reaction monitoring.
to MPAG. In these patients, the observed serum MPA may be below the desired therapeutic concentration of 1–3.5 mg/L, whereas MPAG may be >100 mg/L. In patients who have a low hepatic conjugating capacity, the potential for overimmunosuppression exists. In these cases, the trough serum MPA may be above the desired value whereas the concentration of MPAG is lower than anticipated. Thus, the analysis of both total MPA and MPAG in serum may be important, and in some circumstances the determination of free MPA is warranted (3).

Because of its high analytical sensitivity and specificity, HPLC combined with mass spectrometry has been applied to the quantification of many drugs, including the other immunosuppressants cyclosporine, tacrolimus, and sirolimus. Several HPLC–tandem mass spectrometry (MS/MS) methods for monitoring MPA, free MPA, and MPAG in serum or plasma have been reported. Willis et al. (4) analyzed free MPA by liquid chromatography–atmospheric pressure chemical ionization mass spectrometry with indomethacin as the internal standard. For this assay, the total chromatographic time was ~12 min. Using a structural analog of MPA as internal standard, Atcheson et al. (5) developed a liquid chromatography–electrospray ionization method for monitoring free MPA in plasma. Patel et al. (6) have described a HPLC-MS/MS assay for monitoring free MPA, with a total run time of ~7 min. Although total MPA or MPAG could theoretically be measured by this LC-MS/MS method, some ion suppression was noted at the retention time for MPAG, and the authors only validated a separate 18-min HPLC-UV assay for total MPA and MPAG. Streit et al. (7) have published a sensitive column-switching method for free and total MPA in plasma. Their chromatographic run time is a short 4 min. The only potential limitation to measuring MPAG with this assay is the presence of some ion suppression at 2.5–2.8 min, the point at which MPAG elutes, although the authors here did not purport to measure MPAG with this assay. Recently, Premaud et al. (8) reported a HPLC-MS/MS assay for monitoring both MPA and MPAG, using protein precipitation for sample preparation and indomethacin as an internal standard, with a total chromatographic time of 6 min.

Because of the desire to simultaneously measure both total MPA and MPAG, we have also developed and validated an HPLC-MS/MS method for both of these compounds. Because we can currently analyze cyclosporine, tacrolimus, and sirolimus on the same HPLC-MS/MS system, after solid-phase extraction (SPE) with a common 1-mL cartridge, our goal was to use the same SPE cartridge and mobile phase components as part of this new assay for MPA and MPAG (9).

**Materials and Methods**

**REAGENTS, DRUGS, SERUM CALIBRATORS, AND SERUM CONTROLS**

Methanol, acetonitrile, ammonium acetate, hydrochloric acid, and formic acid were purchased from Fisher Scientific. For SPE, we obtained 25-mg, 1-mL Bond Elut LMS styrene divinylbenzene cartridges from Varian.

MPA was obtained from Roche Bioscience and A.G. Scientific, MPAG was from Roche Bioscience and Analytical Services International, and the internal standard mycophenolic acid carboxybutyloxy ether (MPAC) was a gift from Roche Diagnostics GmbH. These powders were used to make working methanolic solutions for calibrator preparation containing 0.2 g/L MPA or 5.0 g/L MPAG. Using immunosuppressant-free donor serum, we prepared three serum calibrators (0.0/0.0, 4.0/50.0, and 16.0/200.0 mg/L MPA and MPAG, respectively) for the assay, and extracted the calibrators with each run. Using independently prepared methanolic working solutions, we prepared two serum controls containing low (2 mg/L MPA and 25 mg/L MPAG) or high (8 mg/L MPA and 120 mg/L MPAG) drug concentrations.

**SAMPLE PREPARATION AND SPE**

For each calibrator, control, or patient serum specimen, 50 μL of sample was placed in an appropriately labeled 1.5-mL polypropylene microcentrifuge tube, followed by 50 μL of the internal standard solution (20 mg/L MPAC in 0.01 mol/L HCl). A 1.0-mL aliquot of 0.1 mol/L HCl was then added to each tube, and the contents were vortex-mixed for 5 s. The contents of the microcentrifuge tube were then transferred to the Varian SPE column, which had been preconditioned with 1 mL of methanol followed by 1 mL of 0.1 mol/L HCl. A vacuum was applied to the manifold containing the SPE columns, and the samples were fully passed through the cartridge at a rate of ~1 mL/min. The columns were then washed twice with 1 mL of 0.1 mol/L HCl and air dried under reduced pressure for 30 s. MPA, MPAG, and MPAC were eluted from the columns into 10 × 75 mm glass tubes by 750 μL of acetonitrile–water (70:30 by volume). After a vortex-mix, 50 μL of the SPE extract was placed in a 2-mL autosampler vial and further diluted by the addition of 500 μL of water. A 5-μL volume was injected into the HPLC-MS/MS system.

**HPLC-MS/MS ANALYSIS**

The HPLC-MS/MS system consisted of an Agilent 1100 binary pump, well plate sampler, and column heater, interfaced to a triple quadrupole mass spectrometer (Waters Quattro Micro). Gradient chromatographic separation of MPA, MPAG, and MPAC was performed on a 20 × 2.1 (i.d.) mm Waters Atlantis dC18 column (5-μm particle size) maintained at a temperature of 50 °C. The gradient program, flow rate, mobile phase composition, and mass detector tune settings are shown in Table 1. A linear gradient from 10% to 60% B was programmed between 0 and 5 min, and the gradient was held at 60% B for 1.2 min. At 6.3 min, the mobile phase mixture was switched back to the initial conditions, and the run was terminated at 7.0 min. After each run, there is a 40-s sampling and loading time for the Agilent 1100 well plate.
MPAC, calculated with MassLynx 4.0 software.

Integrated peak area ratios of MPA/MPAC or MPAG/MPAC were determined by taking these same control sera through the described extraction protocol and comparing the HPLC-MS/MS responses, corrected for measured volume losses or changes during the extraction, with those obtained for injections of the same volume of pure drugs in methanol–water (50:50), which had also been diluted by the same factor.

**Method Comparison**

The HPLC-MS/MS method proposed here was compared with a HPLC-UV method performed at a national reference laboratory. This HPLC-UV method involves direct measurement of MPA and analysis of serum treated with glucuronidase to measure total hydrolyzable MPA. The difference is reported as the calculated MPAG. For the comparison, aliquots from 37 patient serum specimens being sent by our institution for analysis by the reference laboratory were deidentified and then analyzed by the HPLC-MS/MS method described here.

**Ion Suppression**

Ion suppression was evaluated using a 5 μL/min continuous postcolumn infusion of a solution containing MPA, MPAG, and MPAC. The targeted signal intensities during infusion were those that would be observed from a serum containing 4 mg/L MPA and 10 mg/L MPAG. During the infusion, extracts from three different sera from patients not receiving mycophenolate mofetil were subjected to HPLC-MS/MS analysis. The individual MRM transitions for the three compounds were monitored. For comparison, deionized water was also injected and analyzed.

**Statistical Analysis**

We used simple linear regression analysis to compare the results obtained by HPLC-MS/MS and HPLC-UV. Bland–Altman difference plots were prepared by plotting the difference between the two methods against the mean value obtained by the two methods.
Results

The examples of extracted ion chromatograms for MPA, MPAG, and MPAC shown in Fig. 1 illustrate that these compounds are well separated by HPLC. Any in-source fragmentation, as has been reported to occur for MPAG, would not interfere with the quantification of MPA because MPA and MPAG were separated by 1.2 min.

Shown in Fig. 2 is a comparison of results obtained by HPLC-UV and HPLC-MS/MS methods for the 37 patient specimens. For MPA, the number of comparisons was 33 because 4 specimens had MPA concentrations below the reporting limit of the HPLC-UV method. The regression line for MPAG was affected by a single discrepant result (included in the data in Fig. 2) with HPLC-MS/MS and HPLC-UV results of 152 and 181 mg/L, respectively. Both values represent abnormally high MPAG concentrations.

The ion suppression profiles for a postcolumn infusion of MPA, MPAG, and MPAC into the HPLC eluate of an extracted serum specimen from a patient not receiving mycophenolate mofetil are shown in Fig. 3. No ion suppression was observed for MPA, MPAG, or MPAC when multiple patient serum extracts were tested. The signal intensities and noise profiles for all three compounds were similar to those of a water injection.

The precision data for the HPLC-MS/MS assay are summarized in Table 2. The between-run data were generated from separate consecutive runs performed over a 45-day period. The precision at both low and high concentrations of MPA and MPAG was within the suggested method validation criteria from the consensus panel report for therapeutic drug monitoring of MPA (14). The extraction efficiencies for MPA and MPAG were 99% for both compounds (absolute recoveries slightly less because of loss during sample transfer steps). The HPLC-MS/MS method was linear from 0.1 to 16.0 mg/L for MPA and from 1 to 200 mg/L for MPAG. The lower limit of quantifications were 0.1 mg/L for MPA and 1 mg/L for MPAG for 50 μL of serum and an injection volume of 5 μL.

Both MPA and MPAG were stable during the extraction procedure used for the assay (Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol51/issue5/). We observed no hydrolysis of MPAG to MPA at MPAG concentrations as high as 200 mg/L.

Discussion

A concern in the analysis of MPA by electrospray ionization MS is the reported susceptibility of MPAG to in-source fragmentation, which can lead to loss of the glucuronide moiety and the production of MPA (15). Chromatographic separation of MPA and MPAG thus is recommended so that any artifact-related MRM signal for MPA is not confused with the true signal from MPA in the extract. Interestingly, under the MS conditions used in our assay, we observed essentially no in-source fragmentation of MPAG (Fig. 1) at 25 or 200 mg/L. Compared with the

Fig. 1. Extracted ion chromatograms for an extract of a serum containing 2 mg/L MPA and 25 mg/L MPAG.

Total integration time was 6.5 min. The y axes are scaled to 100% for the largest peak for the specified mass transitions. The number below the peak identifier is the retention time, and full-scale signal intensity is listed in the upper right below the MRM m/z values.
conditions reported by Vogeser et al. (15), we used a lower capillary and cone voltage for our assay and also monitored the ammonium vs hydrogen adducts. There are, however, several potential factors that could account for the difference in the observed in-source fragmentation, such as instrument settings, instrument design, source and desolvation temperatures, and mobile phase composition. Although we have separated MPAG and MPA by 1.2 min and have also performed our chromatography in <6.5 min, the chromatographic time could be further reduced if no significant MRM artifacts occur.

Because of the significance of ion suppression in electrospray ionization MS, we wanted to be certain that the integration and quantification of MPA, MPAG, and MPAC were not affected by any co-eluting serum components. We have verified that, with the extraction protocol, chromatographic separation, and MS conditions selected for our assay, no ion suppression is observed.

During assay development, we observed that the drugs of interest could be eluted from the SPE cartridges with 500 mL/L acetonitrile. However, to be sure that complete elution would always occur and that any minor day-to-day changes in the composition of the elution solvent did not affect the performance of the assay, we chose 700 mL/L acetonitrile as the elution solvent. The fact that we did not use a full 100% organic solvent, which would have eluted all compounds from the SPE cartridge, also may have contributed to the lack of ion suppression for the assay.

In our laboratory, we perform assays for MPA, sirolimus, tacrolimus, and cyclosporine on the same HPLC-MS/MS instrument. The SPE cartridge and mobile phases used for the MPA assay described here are identical to those we use for the other immunosuppressants (9). Because of this, the only minor change needed for the analysis of MPA is a switch of analytical columns from a
Phenomenex C18 to the Waters Atlantis column. No time-consuming priming, equilibration, or flushing of the system is necessary.

**Table 2. CVs for MPA and MPAG.**

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<th>Within run, % (n = 6)</th>
<th>Between runs, % (n = 24)</th>
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<tbody>
<tr>
<td>MPA</td>
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<tr>
<td>2.0 mg/L</td>
<td>3.1</td>
<td>5.3</td>
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<td>8.0 mg/L</td>
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<td>120 mg/L</td>
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<td>4.9</td>
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**References**

4. Willis C, Taylor PJ, Salm P, Tett SE, Pillans PI. Quantification of free mycophenolic acid by high-performance liquid chromatogra-


