Quantification by Liquid Chromatography Tandem Mass Spectrometry of Mycophenolic Acid and Its Phenol and Acyl Glucuronide Metabolites, Gunnar Brandhorst, Frank Streit, Sandra Goetze, Michael Oellerich, and Victor William Armstrong (Universitätssklinikum Göttingen, Abteilung Klinische Chemie/Zentrallabor, 37075 Göttingen, Germany; *address correspondence to this author at: Universitätssklinikum Göttingen, Abteilung Klinische Chemie/Zentrallabor, 37075 Göttingen, Germany; fax 49-551-39-12771, e-mail gunnar.brandhorst@med.uni-goettingen.de)

Background: We developed and validated a rapid and reliable liquid chromatography–tandem mass spectrometry (LC-MS/MS) procedure for the quantification of mycophenolic acid (MPA) and its phenol glucuronide (MPAG) and acyl glucuronide (AcMPAG) metabolites.

Methods: We performed protein precipitation on all samples (calibrators, quality controls, and patient samples) and then subjected them to online solid-phase extraction followed by reversed-phase liquid chromatography for 4.0 min. The carboxybutoxy ether of MPA (MPAC) was used as the internal calibrator. The separated compounds (MPA, MPAG, AcMPAG, and MPAC) were detected by electrospray ionization-coupled MS/MS. We compared LC-MS/MS results with results for the same samples obtained with a validated HPLC procedure with an ultraviolet detector.

Results: Comparison with the validated HPLC-ultraviolet procedure demonstrated good agreement. The Passing–Bablok regression was $y = 0.968x − 0.058$ for MPA, $y = 1.08x − 1.697$ for MPAG, and $y = 0.952x + 0.076$ for AcMPAG. Assay imprecision showed a CV $<10\%$ at 3 concentrations for each compound. The lower limit of quantification was 0.1 mg/L for MPA, 1.0 mg/L for MPAG, and 0.05 mg/L for AcMPAG. The mean analytical recovery was 90%–110%. The assay was linear from 0.1 to 50 mg/L for MPA ($r = 0.9987$), from 1 to 500 mg/L for MPAG ($r = 0.9999$), and from 0.05 to 10 mg/L for AcMPAG ($r = 0.9988$). Quantification of the compounds was not affected by in-source fragmentation or ion suppression.

Conclusion: The LC-MS/MS assay described here is valid and reliable for the quantification of total MPA, MPAG, and AcMPAG in serum.

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Mycophenolic acid acyl glucuronide (AcMPAG), a secondary metabolite of mycophenolic acid (MPA), has pharmacologic and toxicologic properties that may contribute to the gastrointestinal side effects such as diarrhea that are associated with MPA (1–3). For >7 years we have used HPLC with an ultraviolet detector (HPLC-UV) in our laboratory to measure MPA and its metabolites (4,5). Although we rarely observed interference with MPA and its inactive phenol glucuronide metabolite (MPAG), we recently found that, because of interference, AcMPAG could not be quantified in ~26% of samples. Therefore, we developed and validated an LC-MS/MS assay for MPA, MPAG, and AcMPAG that was based on our previously published assay (6).

We purchased HPLC-grade methanol, acetonitrile, acetic acid, ammonium acetate, sodium tungstate, and perchloric acid from Merck. MPA, MPAG, AcMPAG, and the carboxybutoxy ether of MPA (MPAC) were kind gifts from Roche Pharmaceuticals.

We prepared the following stock solutions: 3.0 g/L AcMPAG in dimethyl sulfoxide, 1.0 g/L MPA in acetonitrile, 5.0 g/L MPAG in acetonitrile and water (80 mL acetonitrile, 20 mL water), and 1.0 g/L of MPAC in acetonitrile. The stock solutions were prepared twice (for calibrators and quality controls) and stored at 4 °C (AcMPAG) or −20 °C (MPA, MPAG, and MPAC). We prepared 2 serum calibrators each for MPA (3.0 and 10.0 mg/L), MPAG (10.0 and 100.0 mg/L), and AcMPAG (1.0 and 3.0 mg/L). Because of the inherent instability of AcMPAG at physiologic pH, the AcMPAG calibrators were prepared by adding stock solution to EDTA-plasma from healthy blood donors (who gave informed consent), acidified with orthophosphoric acid (14.5 g/L). Serum quality control samples were prepared in drug-free serum (0.2, 1.0, and 25.0 mg/L MPA and 10.0, 50.0, and 250.0 mg/L MPAG; Bio-Rad) or acidified plasma (0.1, 0.6, and 1.5 mg/L AcMPAG).

Sample preparation was similar to our previous protocol (6), except that the volumes were decreased: 100 μL of calibrator, quality control or patient sample (EDTA plasma) was treated with 10 μL of perchloric acid (150 g/L), 10 μL sodium tungstate (250 g/L), and 50 μL of internal calibrator solution (15 mg/L MPAC in acetonitrile). After vigorous mixing and centrifugation (5 min at 14 000g), the supernatants were transferred into 500-μL polypropylene tubes (Eppendorf), and 5 μL was injected into the chromatographic system. For quantification of MPAG, the samples were diluted 1:10 with drug-free serum before extraction.

The chromatographic system consisted of a binary pump, a quaternary pump, an autosampler with a 200-μL sample loop, a decreased pressure degasser, and a Peltier column oven (all Series 200, Perkin-Elmer). The analytical column was an Aqua Perfect 150 × 4 mm C18 reversed-phase column, 5 μm particle size (MZ-Analysentechnik). A Waters Oasis HLB Extraction Column (25 μm, 2 × 15 mm) served as precolumn for online solid-phase extraction. The compounds were injected onto the precolumn in water (pH 3.0 with acetic acid) at 3 mL/min. After 0.4 min, the samples were eluted and separated on the analytical column, maintained at 30 °C (1.3 mL/min; 520 mL methanol, 170 mL acetonitrile, 310 mL water, 0.2 mmol/L ammonium acetate, and 0.244 g/L formic acid, pH 3.0). For washing and equilibration, the injection pump was operated as follows: 0.0 min 3.0 mL/min (water, pH 3.0), 2.0 min 1.0 mL/min (methanol), 3.0 min 2.0 mL/min (methanol), 3.0 min 5.0 mL/min (water, pH 3.0), 4.0 min 3.0 mL/min (water, pH 3.0). The valve was switched at 0.0 min to B (injection), at 0.4 min to A
(elution), at 2.5 min to B, at 3.0 min to A, and at 3.5 min to B.

A PE SCIEX API 2000 triple-quadrupole mass spectrometer with a turbo-ion spray interface was used for detection. Conditions were as follows: split 1:10; positive ion spray 5000 V; source 250 °C; 50 psi for ion-source gas; 70 psi for ion source gas 2; entrance potential 8.5 V; declustering potential 20 V; and collision energies of 30 eV (MPAC), 25 eV (MPA), and 35 eV (MPAG and AcMPAG). Multiple-reaction monitoring was performed for the ammonium adducts and the product ions of MPAC (m/z 438.0/207.0) and MPA (m/z 338.1/207.1) and the metabolites AcMPAG and MPAG (m/z 514.2/207.1). Drug concentrations were quantified through the peak area ratios.

For examples of the ion chromatograms for MPA, MPAG, and AcMPAG, see Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol52/issue10. In-source fragmentation occurred with ~1% of MPAG, but because of chromatographic separation, this MPAG fragmentation did not affect MPA quantification. The retention times were 2.75 min for MPA, 1.8 min for MPAG, 2.2 min for AcMPAG, and 2.7 min for MPAC.

The assay was validated in accordance with Food and Drug Administration guidelines (7) (Table 1). Within-run and between-run imprecision were acceptable (CV, <10%; n = 15) for each compound and each quality control. The lower limit of quantification (defined as the concentration at which the CV does not exceed 20%; n = 15) was 0.1 mg/L for MPA, 1.0 mg/L for MPAG, and 0.05 mg/L for AcMPAG. The mean analytical recovery (n = 15 for each quality control) was 90% to 110%. The assay was linear from 0.1 to 50 mg/L for MPA (r = 0.9987), from 1 to 500 mg/L for MPAG (r = 0.9999), and from 0.05 to 10 mg/L for AcMPAG (r = 0.9988). Assessment of carryover effects showed <0.5% for each compound (0.41% after 10 mg/L MPA, 0.23% after 100 mg/L MPAG, 0.06% after 3 mg/L AcMPAG; each n = 10). Mean extraction efficiencies were 81% for MPA and 107% for MPAG and AcMPAG (each n = 4). Lower extraction efficiency for MPA compared with its metabolites was consistent with previous results (4). Evaluation of matrix effects through a postcolumn infusion experiment (8) showed that suppression of the ion generation occurred only at 0.7–1.0 min (see Fig. 2 in the online Data Supplement). No ion suppression was present during the elution times for the compounds of interest (1.5–3.5 min).

To compare our LC-MS/MS method with a validated HPLC-UV method (4, 5), we analyzed specimens obtained from routine analysis of mainly kidney transplantation patients (n = 46 for MPA and MPAG; n = 28 for AcMPAG). Regression analysis was performed by use of the nonparametric procedure of Passing and Bablok (9) and EVAPAK 3.1 (Roche Diagnostics) software (Fig. 1). The comparison showed excellent agreement for MPA and MPAG. In addition, the use of proficiency testing scheme samples and the method mean data (mean deviation, 5.8%; n = 20) (10) showed good congruence between the MPA concentrations determined with our LC-MS/MS method. For AcMPAG measurements, we observed a slight bias at concentrations <0.3 mg/L, for which the LC-MS/MS yielded higher results, probably attributable to the relatively low signal-to-noise ratio at low AcMPAG concentrations in the HPLC-UV assay, leading to increasing impairment by frequent interferences, which are not always easy to identify.

At physiologic pHs, AcMPAG has been found to undergo degradation (4) attributed to different reagents used for sample preparation (11). To verify the degradation of AcMPAG in plasma before and after precipitation of the samples, we supplemented EDTA-plasma with AcMPAG and compared the concentrations during various incubation times with and without acidification. The storage of plasma samples at ambient temperature without prior acidification led to a degradation of >50% of AcMPAG after 44 h, whereas almost no degradation occurred in acidified samples (see Fig. 3 in the online Data Supplement). In contrast, after precipitation, the AcMPAG concentration remained stable for at least 44 h even at 4 °C, regardless of any acidification before preparation. Therefore, we can confirm the results of Shipkova et al. (5) indicating that prior sample acidification has a substantial effect on the stability of AcMPAG in plasma samples and thus is essential for AcMPAG quantification.

Many assays have been described that use HPLC-UV (11, 12, 13) or mass spectrometry (6, 14, 15) for the quantification of MPA and/or MPAG. The use of HPLC-UV methods to measure AcMPAG has been reported (11, 12), but the use of mass spectrometry has not. Advantages of this LC-MS/MS assay for routine use include the small sample volume requirement (100 μL) and ease of sample preparation. Chromatographic separation decreases in-source fragmentation of MPAG, which can lead to false-high MPA concentration results (16). The assay is not impaired by ion suppression during compound detection. Problems with AcMPAG quantification caused by interferences in the HPLC-UV assay can be overcome by specific mass detection of the compound. Furthermore, the assay time allows a maximum throughput of 240 samples per day and system. We found this speed of

| Table 1. Assay validation: analytical recovery and imprecision. |
|-----------------|------------------|------------------|------------------|
| mg/L | Within-run CV (n = 15), % | Between-run CV (n = 15), % | Recovery (n = 15), % |
| MPA | 0.2 | 8.5 | 7.0 | 110 |
| | 1.0 | 5.5 | 6.8 | 100 |
| | 25.0 | 4.2 | 5.5 | 91 |
| MPAG | 10.0 | 5.5 | 6.5 | 100 |
| | 50.0 | 4.7 | 6.0 | 100 |
| | 250.0 | 6.2 | 8.1 | 96 |
| AcMPAG | 0.1 | 9.8 | 8.4 | 98 |
| | 0.6 | 6.6 | 7.2 | 96 |
| | 1.5 | 5.0 | 6.7 | 96 |
processing advantageous because of the increasing number of requests for the quantification of MPA and its metabolites for routine use and clinical trials.

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References


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