Determination of the Acyl Glucuronide Metabolite of Mycophenolic Acid in Human Plasma by HPLC and Emit

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Background: The acyl glucuronide (AcMPAG) of mycophenolic acid (MPA) has been found to possess pharmacologic and potentially proinflammatory activity in vitro. To establish its pharmacologic and toxicologic relevance in vivo, a reversed-phase HPLC method was modified to simultaneously determine MPA, the phenolic MPA-glucuronide (7-O-MPAG), and AcMPAG. In addition, cross-reactivity of AcMPAG in the Emit assay for MPA was investigated.

Methods: The procedure used simple sample preparation, separation with a Zorbax Eclipse-XDB-C8 column, and gradient elution. AcMPAG was quantified as 7-O-MPAG-equivalents.

Results: The assay was linear up to 50 mg/L for MPA, 250 mg/L for 7-O-MPAG, and 10 mg/L for AcMPAG ($r > 0.999$). Detection limits were 0.01, 0.03, and 0.04 mg/L for MPA, 7-O-MPAG, and AcMPAG, respectively. The recoveries were 99–103% for MPA, 95–103% for 7-O-MPAG, and 104–107% for AcMPAG. The within-day imprecision was <5.0% for MPA, <4.4% for 7-O-MPAG, and ≤14% for AcMPAG (0.1–5 mg/L). The between-day imprecision was <6.2%, <4.5%, and ≤14% for MPA, 7-O-MPAG, and AcMPAG, respectively. When isolated from microsomes, purified AcMPAG (1–10 mg/L) revealed a concentration-dependent cross-reactivity in an Emit assay for the determination of MPA ranging from 135% to 185%. This is in accordance with the bias between HPLC and Emit calculated in 270 samples from kidney transplant recipients receiving mycophenolate mofetil therapy, which was greater (median, 151.2%) than the respective AcMPAG concentrations determined by HPLC. AcMPAG was found to undergo hydrolysis when samples were stored up to 24 h at room temperature or up to 30 days at 4 °C or −20 °C. Acidified samples (pH 2.5) were stable up to 30 days at −20 °C.

Conclusions: The HPLC and Emit methods for AcMPAG described here may allow investigation of its relevance for the immunosuppression and side effects associated with mycophenolate mofetil therapy.

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Mycophenolic acid (MPA),¹ the immunosuppressive metabolite of mycophenolate mofetil (MMF), is increasingly used for immunosuppressive therapy after solid-organ transplantation, and also has been proposed for therapy of several autoimmune diseases (1–3). MPA is metabolized primarily by glucuronidation at the phenolic hydroxyl group to form the 7-O-glucuronide conjugate (7-O-MPAG), which is the major urinary excretion product of the drug (1, 3). We recently characterized two further metabolites of MPA, including an acyl glucuronide metabolite (AcMPAG), in the plasma of liver, kidney, and heart transplant recipients receiving MMF (4). This metabolite is also formed in vitro by microsomal preparations from human liver, kidney, and intestinal tissue (5) and was found to possess a concentration-dependent pharmacologic potency comparable to that of the parent drug (6). Furthermore, AcMPAG demonstrated a potential to induce cytokine release and cytokine mRNA expression in human mononuclear leukocytes (7), which may be of relevance with respect to side effects observed in patients receiving MMF therapy. In addition, acyl glucuronides are known to be reactive metabolites of

¹ Nonstandard abbreviations: MPA, mycophenolic acid; MMF, mycophenolate mofetil; 7-O-MPAG, 7-O-glucuronide of mycophenolic acid; AcMPAG, acyl glucuronide of mycophenolic acid; MPAC, carboxybutoxy ether of MPA; UV, ultraviolet; and $d_k$, critical difference.
drugs bearing a carboxylic acid function and to possess toxic potential either by direct tissue damage as a result of covalent binding to proteins, lipids, and nucleic acids or by the formation of adducts with proteins, leading to hypersensitivity or idiosyncratic drug reactions (8,9).

Although the latter has not yet been demonstrated for AcMPAG, the possibility exists that this acyl glucuronide contributes to side effects observed in patients receiving MMF therapy through the mechanisms mentioned above. Determination of AcMPAG plasma concentrations may therefore be of importance for the monitoring of MMF therapy with regard to its pharmacologic and toxicologic effects. To investigate these effects, appropriate analytic methods are needed. We have modified our recently published HPLC method for the simultaneous determination of MPA and 7-O-MPAG (10) to also monitor AcMPAG.

We found that AcMPAG cross-reacts with the MPA antibody used in the commercially available Emit assay (Dade-Behring). This could explain the 5–40% overestimation of Emit MPA values compared with HPLC in different transplant recipients (11–13). The overestimation was found to correlate with the AcMPAG plasma concentrations determined with a different HPLC procedure (14). Establishing the extent of the cross-reactivity of AcMPAG in the Emit assay for MPA is therefore a prerequisite for the interpretation of Emit test results with respect to the monitoring of MMF therapy.

Acyl glucuronides are known to readily undergo hydrolysis to the parent drug under neutral or slightly alkaline conditions, with the rate of hydrolysis being dependent on the temperature (8,9,15). Furthermore, they can accumulate in the blood of patients with renal failure, and in vitro hydrolysis in plasma samples obtained from such patients could compromise the quantification of both the metabolite and the parent compound. Therefore, we investigated the stability of AcMPAG in patient plasma samples. To improve the accuracy of the analysis, we evaluated the possibility of stabilizing samples according to the procedure recommended by Hyneck et al. (15) for tolmetin acyl glucuronide.

Materials and Methods

DRUGS AND REAGENTS

MPA, 7-O-MPAG, and the internal standard, carboxybutoxy ether of MPA (MPAC), were a gift from Hoffmann-La Roche (Grenzach-Wyhlen, Germany). AcMPAG was generated using human liver microsomes, and was isolated and purified according to a previously published procedure (4). Sodium tungstate dihydrate, potassium dihydrogen phosphate, sodium hydroxide, phosphoric acid, and perchloric acid were from Merck. Acetonitrile (HPLC grade) was obtained from J.T. Baker. Stock solutions of MPA and of the internal standard MPAC were prepared separately in acetonitrile, each at a concentration of 1 g/L, and stored at −20 °C. The 7-O-MPAG stock solution (5 g/L) was prepared in acetonitrile-water (80:20, by volume) and stored at −20 °C. A stock solution of AcMPAG was prepared in deionized sterile water, acidified with 0.3 g/L phosphoric acid to yield pH 2, and stored at −20 °C for up to 3 weeks. The concentration of the AcMPAG stock solution was 1 g/L as determined in 7-O-MPAG-equivalents, which were used for quantification because we lacked sufficient amounts of purified AcMPAG to obtain an accurate weight. Furthermore, in contrast to 7-O-MPAG, the acyl glucuronide is not sufficiently stable to prepare calibration material to be stored for extended time periods (8,9). The ultraviolet (UV) spectrum of AcMPAG with characteristic absorption maxima at ~216, 250, and 306 nm is similar to that of 7-O-MPAG (absorption maxima at 215, 252, and 294 nm) (4).

SAMPLE PREPARATION

The sample pretreatment procedure was as described previously (10). Briefly, 200 μL of plasma and 100 μL of acetonitrile containing the internal standard MPAC (15 mg/L) were mixed by vortex-mixing in a 1.5-mL polypropylene tube for 5 s. This was followed by the sequential addition of 20 μL of 250 g/L sodium tungstate and 20 μL of 150 g/L perchloric acid and vortex-mixing for 15 s after each addition. The sample was then centrifuged for 5 min at 10 000 g, and 50 μL of the supernatant was removed for chromatography.

CHROMATOGRAPHIC CONDITIONS

To determine AcMPAG plasma concentrations simultaneously with MPA and 7-O-MPAG concentrations in one HPLC run with favorable performance characteristics for all three analytes, we modified the chromatographic conditions used previously (10). The separation was achieved with a Zorbax Eclipse XDB-C8 column (25 cm × 4.6 mm i.d.; particle size, 5 μm; Hewlett Packard) as stationary phase. This column is able to separate highly polar compounds such as MPA and glucuronide metabolites, and is also stable over the wide pH range required for the method presented here. The mobile phase (flow rate, 1.2 mL/min) consisted of solution A (250 mL acetonitrile and 750 mL phosphate buffer, pH 3.0, 20 mmol/L final concentration) and solution B (700 mL acetonitrile and 300 mL phosphate buffer, pH 6.5, 20 mmol/L final concentration) that formed the following gradient: 0–4.5 min (7% B); 4.5–7 min (34% B); 7–13.5 min (34% B); 13.5–14 min (100% B); 14–17.5 min (100% B); 17.5–18 min (7% B). The column was maintained at 42 °C. The HPLC system consisted of a chromographic pump (M480), an automatic injector (GINA 50), a diode array detector (UV 340S), and a computer interface system controller linked to a PC (Dionex-Gynkotek). The compounds were quantified by absorbance at 215 nm in internal standard mode, using peak-area ratios. The Chromeleon software (Dionex-Gynkotek), Ver. 5.42, was used for recording and calculating the data and also for recording the UV spectra when required. The calibration and quality control were
performed by use of solutions prepared in-house (10), which were analyzed with each run.

**ASSESSMENT OF PERFORMANCE CHARACTERISTICS**

The detection limit of the method was calculated using a signal-to-noise ratio of 3. For this purpose, the noise signal of the baseline was obtained from a segment of the chromatogram that preceded the AcMPAG, MPA, or 7-O-MPAG peak. The limit of quantification was defined according to Shah et al. (16) as the smallest concentration of AcMPAG with a between-day imprecision <20% and a mean inaccuracy <20%. The linearity of the method was established using drug-free plasma with MPA, 7-O-MPAG, and AcMPAG added to yield concentrations of 0.04–50 mg/L for MPA, 1–500 mg/L for 7-O-MPAG, and 0.05–10 mg/L for AcMPAG. The within- and between-run imprecision and extraction efficiency were studied by adding the compounds to drug-free plasma; final concentrations were 0.2, 1, and 25 mg/L MPA; 10, 50, and 250 mg/L 7-O-MPAG; and 0.1, 1 and 5 mg/L AcMPAG.

The extraction efficiency was calculated by comparing peak areas obtained from the extracted plasma samples with MPA, AcMPAG, or 7-O-MPAG added with the peak areas obtained for acetonitrile-water (80:20, by volume) solutions containing the same amount of the compounds, which were injected directly onto the column without extraction. To obtain information on the accuracy of the method, the analytical recovery was determined by adding known amounts of MPA (0.2, 1, and 25 mg/L), 7-O-MPAG (10, 50, and 250 mg/L), and AcMPAG (0.1, 1, and 5 mg/L) to a drug-free plasma pool. The recovery was calculated by comparing the measured concentrations with the expected concentrations.

Potential chromatographic interference by commonly administered drugs was evaluated by analysis of patient specimens received for routine therapeutic drug monitoring, including transplant patients receiving immunosuppressive therapy without MMF, therapeutic drug monitoring quality-control sera (Chiron Diagnostics), and drug standards dissolved in methanol. In addition, the existence of endogenous chromatographic interferences was evaluated by separate analysis of 50 patient specimens free of MPA.

**METHOD COMPARISON**

Fifty-one plasma specimens derived from pharmacokinetic studies in renal, liver, or heart transplant recipients were used to compare the method presented here with the established HPLC procedure published previously (10) with respect to MPA and 7-O-MPAG plasma concentrations.

**ESTIMATION OF ACMPAG CROSS-REACTIVITY WITH THE EMIT MPA ANTIBODY**

The AcMPAG cross-reactivity in the Emit assay was studied with drug-free plasma to which AcMPAG was added to yield final concentrations of 1.25, 2.5, 5, and 10 mg/L. Each solution was divided into two 450-μL portions. To the first portion, 45 μL of NaOH (2 mol/L) was added, and the samples were then left at room temperature for 2 h to achieve complete hydrolysis of AcMPAG to MPA. The pH was then adjusted to 7.4 by the addition of H3PO4 (850 g/L), and the samples were analyzed with the Emit. To verify complete hydrolysis of AcMPAG, an aliquot of each sample was subjected to HPLC analysis. The second portion (450 μL) served as control and was analyzed directly by Emit or HPLC after the volume was corrected using doubly distilled water. To investigate whether the immunologic reactivity was affected by the pH changes during the hydrolysis procedure, the same protocol was applied to solutions to which matching MPA concentrations instead of AcMPAG had been added. All experiments were performed in duplicate and repeated on another day.

To examine the effect of MPA on the cross-reactivity of the metabolite AcMPAG in the immunoassay, plasma samples (n = 2) containing either 2.5 or 5 mg/L AcMPAG were supplemented with 1 and 5 mg/L MPA, respectively, and treated according to the protocol above. In addition, 270 samples collected from kidney transplant recipients receiving MMF therapy were investigated in parallel, using HPLC and Emit, and the absolute differences in the results from both techniques were compared to the respective AcMPAG concentrations determined by HPLC.

**STABILITY OF ACMPAG IN HUMAN PLASMA AS DETERMINED BY HPLC**

To investigate the stability of AcMPAG, two plasma pools from patients receiving MMF therapy as well as two drug-free plasma pools supplemented with AcMPAG were used. These pools contained between 0.7 and 4.09 mg/L AcMPAG. An aliquot from each pool was acidified using phosphoric acid (850 g/L) to pH 2.5. The resulting pools were divided into 200-μL aliquots, which were split into three parts and stored separately at −20 °C, 4 °C, or room temperature. AcMPAG concentrations were determined immediately after preparation of the pools and after 1 day, 7 days, and 30 days of storage for the samples kept at −20 °C and 4 °C. Samples stored at room temperature were analyzed immediately after preparation of the pools and after 1, 2, 5, 12, and 24 h of storage.

**STATISTICS**

The nonparametric regression procedure of Passing and Bablok (17) was used for method comparison (EVAPAK, Ver. 2.08; Boehringer Mannheim). For correlation analysis, Spearman correlation coefficient was calculated. To establish the stability of AcMPAG, critical differences (d*) were calculated according to the formula $d^* = 2 \times \sqrt{2} \times S$, with $S$ being the standard deviation of the method from day to day. Values were considered significantly different if the absolute difference between two values, $x_1$ and $x_2$, was greater than $d^*_k (|x_1 - x_2| > d^*_k)$ (18).
**Results**

**HPLC procedure**

Representative chromatograms of a plasma sample containing 1.25 mg/L MPA, 1.85 mg/L AcMPAG, and 149.0 mg/L 7-O-MPAG, obtained from a kidney recipient 6 h after the patient received 0.6 g/m² of MMF, and a sample obtained from a renal transplant patient not receiving MMF therapy are shown in Fig. 1, A and B, respectively. The retention times of 7-O-MPAG, AcMPAG, MPAC, and MPA were 4.2, 7.8, 12.5, and 13.4 min, respectively.

No interferences were observed from endogenous compounds and various drugs that are potentially coadministered with MMF, such as acetaminophen, amikacin, amoxicillin, amphotericin B, caffeine, carbamazepine, cefazolin, chloramphenicol, cimetidine, clonazepam, cyclosporin A, diazepam, digoxin, dopamine, gentamicin, lidocaine, netilmicin, phenobarbital, phenytoin, prednisolone, prednisone, primidone, rapamycin, salicylate, tacrolimus, theophylline, tobramycin, and vancomycin.

Linearity was verified up to 50 mg/L for MPA, up to 250 mg/L for 7-O-MPAG, and up to 10 mg/L for AcMPAG (correlation coefficients, \( r > 0.999 \)).

The within- and between-run imprecision and the analytical recovery for AcMPAG are given in Table 1. For AcMPAG, the detection limit was 0.04 mg/L, the lower limit of quantification was 0.1 mg/L, and the extraction efficiency was 72–78% (\( n = 3 \)) in the working range (0.1–5 mg/L). Because the sample pretreatment procedure was the same as that published previously (10), the performance characteristics for MPA and 7-O-MPAG remained similar. In the working range for MPA (0.2–25 mg/L), the within-run imprecision was 2.0–5.0% (\( n = 10 \)), the between-run imprecision was 3.4–6.2% (\( n = 10 \)), the analytical recovery was 99–103% (\( n = 5 \)), and the extraction efficiency was 56–60% (\( n = 3 \)). In the working range for 7-O-MPAG (10–250 mg/L), the within-run imprecision was 2.3–4.4% (\( n = 10 \)), the between-run imprecision was 3.6–4.5% (\( n = 10 \)), the analytical recovery was 95–103% (\( n = 5 \)), and the extraction efficiency was 73–78% (\( n = 3 \)).

The detection limits (signal-to-noise ratio = 3) were 0.01 mg/L for MPA and 0.03 mg/L for 7-O-MPAG.

The procedure requires 10 min for a single sample preparation, 30 min for preparation of a batch of 20 samples, and 21 min for a single chromatographic development.

Excellent agreement was observed between plasma MPA (\( r = 0.998; y = 1.019 (0.998–1.045)x - 0.006 (–0.065 to 0.065) \); 68% median distance = 0.159; \( S_{\mu y} = 0.26; n = 51 \)) and 7-O-MPAG (\( r = 0.998; y = 1.031 (1.0–1.063)x + 0.313 (–1.37 to 1.0) \); 68% median distance = 2.51; \( S_{\mu y} = 2.69; n = 51 \)) concentrations determined by the new (\( y \)) and the original procedure (\( x \); Fig. 2).

In 406 patient samples obtained during pharmacokinetic investigations, AcMPAG concentrations were 0.05–5.4 mg/L with a median of 0.75 mg/L.

**Cross-reactivity of AcMPAG in Emit assay**

As shown in Table 2, values obtained with the Emit assay in plasma samples supplemented with AcMPAG were 135.3–184.8% higher when compared with values observed with the same samples after hydrolysis of AcMPAG to MPA using NaOH. Furthermore, the cross-reactivity of AcMPAG showed a concentration-dependent increase in the Emit. The completeness of hydrolysis was verified by HPLC. The hydrolysis procedure itself had no influence on the immune reaction as shown with control

![Fig. 1. Chromatograms of a plasma sample from a renal transplant recipient on MMF therapy (0.6 g/m² twice per day) obtained 6 h after the last dose, containing 1.23 mg/L MPA, 1.85 mg/L AcMPAG; and 149 mg/L 7-O-MPAG (A); and a plasma sample obtained from a renal transplant recipient not receiving MMF therapy (B). mAU, milliabsorbance units. (A), MPAC (15 mg/L) was added to the plasma sample as internal standard (IS).](image-url)

**Table 1. Imprecision (CVs) and analytical recovery of AcMPAG with the HPLC method.**

<table>
<thead>
<tr>
<th>AcMPAG added to drug-free plasma, mg/L</th>
<th>CV, %</th>
<th>Analytical recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Within-run (n = 10)</td>
<td>Between-run (n = 10)</td>
</tr>
<tr>
<td>0.1</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>1</td>
<td>5.0</td>
<td>5.6</td>
</tr>
<tr>
<td>5</td>
<td>3.8</td>
<td>4.7</td>
</tr>
</tbody>
</table>

* Analytical recovery given as mean (SD).*
experiments in which AcMPAG was replaced by MPA (Table 2).

MPA, when added to two plasma pools containing either 2.5 or 5 mg/L AcMPAG, did not influence the cross-reactivity of AcMPAG in the Emit assay. After the addition of 1 mg/L MPA to these pools, mean cross-reactivities of 149.3% and 165.9% were observed (n = 2). With 5 mg/L MPA, the cross-reactivities were 154.4% and 166.0%, respectively (n = 2).

Correlation analysis (Fig. 3) between AcMPAG concentrations measured by HPLC and the bias between MPA concentrations determined by Emit and HPLC (MPA_{Emit} - MPA_{HPLC}) in 270 patients samples revealed an $r$ of 0.88. The interassay bias was in general higher than would be expected from AcMPAG concentrations determined with HPLC (median, 151.2%; 16–84 percentile, 98.5–278.6%). This is in accordance with an increased immunologic cross-reactivity of AcMPAG in the Emit assay as shown above.

**STABILITY OF AcMPAG IN PLASMA SAMPLES**

Storage of plasma pools containing AcMPAG at concentrations observed in vivo at room temperature produced a significant time-dependent decrease in the concentration of this metabolite (Fig. 4A). Compared with initial values

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**Table 2. Cross-reactivity of AcMPAG in the Emit assay.**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>MPA concentration, mg/L</th>
<th>MPA_{before} - MPA_{after} %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In Emit before hydrolysis (n = 4)</td>
<td>In Emit after hydrolysis (n = 4)</td>
</tr>
<tr>
<td>AcMPAG</td>
<td>1.23 (0.08)</td>
<td>0.91 (0.13)</td>
</tr>
<tr>
<td></td>
<td>2.66 (0.14)</td>
<td>1.78 (0.14)</td>
</tr>
<tr>
<td></td>
<td>5.82 (0.59)</td>
<td>3.67 (0.20)</td>
</tr>
<tr>
<td></td>
<td>13.19 (1.34)</td>
<td>7.14 (0.55)</td>
</tr>
<tr>
<td>MPA</td>
<td>1.05 (0.04)</td>
<td>1.06 (0.08)</td>
</tr>
<tr>
<td></td>
<td>2.00 (0.08)</td>
<td>1.95 (0.04)</td>
</tr>
<tr>
<td></td>
<td>4.19 (0.11)</td>
<td>4.10 (0.3)</td>
</tr>
<tr>
<td></td>
<td>8.42 (0.36)</td>
<td>8.28 (0.24)</td>
</tr>
</tbody>
</table>

*a Concentrations are given as means (SD).

$\frac{\text{MPA}_{\text{before}} - \text{MPA}_{\text{after}}}{\text{MPA}_{\text{after}}} \times 100$.

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Fig. 2. Comparison of MPA (A) and 7-O-MPAG (B) concentrations with the new HPLC method vs the established HPLC method in 51 samples from transplant recipients receiving MMF therapy.

Fig. 3. Spearman correlation analysis of AcMPAG concentrations determined by HPLC and the bias observed between MPA determinations by Emit and HPLC in 270 samples from kidney transplant recipients receiving MMF therapy.

AcMPAG values are given in $\mu$mol/L 7-O-MPAG-equivalents. Experimental details as given in Materials and Methods.
(median, 2.61 mg/L; range, 0.7–4.09 mg/L; n = 4), there was a median AcMPAG decrease of 37.8% (range, 35.9–44.3%; n = 4) after 24 h, producing concentrations between 0.39 and 2.62 mg/L (median, 1.62 mg/L). This decrease was not observed when the plasma pools were adjusted to pH 2.5 before storage (Fig. 4A). When non-acidified samples were stored at 4 °C, there was a significant decrease of AcMPAG, with median concentrations of 1.83 mg/L (range, 0.41–3.43 mg/L; n = 4) on day 1, 1.61 mg/L (range, 0.32–3.13 mg/L; n = 4) on day 7, and 1.01 mg/L (range, 0.23–1.88 mg/L; n = 4) on day 30. This decrease corresponds to a 29.7% median loss of AcMPAG within 1 day (range, 16.1–41.9%; n = 4), 38.4% (range, 23.0–53.9%; n = 4) within 7 days, and 61.4% (range, 54–73.3%; n = 4) within 30 days (Fig. 4B). When nonacidified samples were stored at 220 °C for 7 or 30 days, significant decreases of AcMPAG to median concentrations of 2.30 mg/L (range, 0.6–3.79 mg/L; n = 4) on day 7 and 2.06 mg/L (range, 0.55–3.23 mg/L; n = 4) on day 30 were also observed (Fig. 4B). When acidified samples were stored for >24 h at 4 °C, the AcMPAG concentration was significantly reduced to a median concentration of 2.30 mg/L (range, 0.59–3.77 mg/L; n = 4) on day 7, and a median concentration of 2.22 mg/L (range, 0.53–3.69 mg/L; n = 4) on day 30 (Fig. 4B). In contrast, the metabolite was stable up to 30 days at −20 °C in all acidified samples. A nonsignificant loss of AcMPAG was found up to 24 h in the acidified samples stored at 4 °C as well as in the nonacidified samples stored at −20 °C.

**Discussion**

In this report, we describe a modified HPLC method that allows the simultaneous determination of AcMPAG, MPA, and 7-O-MPAG within one analysis. The method is simple, rapid, and convenient. In addition, the performance characteristics show that it is accurate and reproducible. Because AcMPAG is very unstable in vitro (Fig. 4) and the chemical synthesis is difficult (8), it was quantified as 7-O-MPAG-equivalents. Because of the comparable extraction efficiencies of AcMPAG and 7-O-MPAG and their similar UV spectra, a favorable analytical recovery was obtained (Table 1). When needed, the lower quantification limit for AcMPAG of 0.1 mg/L (between-run imprecision, 14%) can be lowered to 0.05 mg/L by increasing the injection volume. The linearity of the method described was sufficient for >95% of the plasma samples investigated to date. The remaining samples had to be injected either at a reduced volume because of MPAG concentrations exceeding the capacity of the UV detector or at a higher volume because of low AcMPAG concentrations. The low pH used during sample pretreatment is favorable with respect to the stability of AcMPAG. This allows analysis of large series, using an autosampler, because samples can then be kept at room temperature for up to 24 h. More than 600 chromatographic runs can be achieved with one Eclipse XDB-C8 column without any deterioration of the separation performance.

The method was validated by comparison with the original HPLC method (10), which has been used in pharmacokinetic studies on MPA in pediatric and adult renal transplant recipients (19, 20). Very good agreement between the two methods was observed for both MPA and 7-O-MPAG (Fig. 2).

The method is also suitable for the determination of the free MPA fraction after ultrafiltration of the plasma using the protocol described in the original procedure (10) as well as for the determination of the prodrug MMF (retention time, ~9 min; data not shown). This may be of
potential interest in patients receiving MMF through the intravenous route (1, 21).

As described previously by our group (11, 14), the Emit MPA assay also reflects AcMPAG plasma concentrations because of the cross-reactivity of its anti-MPA antibody with AcMPAG. In contrast, 7-O-MPAG shows no cross-reactivity (3, 13) in this assay, which suggests that the epitope recognized by the anti-MPA antibody includes the free hydroxyl group at position seven of the phenol ring of the molecule. Because AcMPAG has pharmacologic activity in vitro (6), it can be speculated that the Emit data may better reflect the immunosuppression during MMF treatment than will values obtained with methods that measure only MPA. However, the interpretation of values determined by this technique is difficult, particularly because of the concentration-dependent cross-reactivity (Table 2). Such increased cross-reactivity may be explained by the complementarity of the binding site of an antibody to different antigens as well as by the flexibility of the binding site to undergo conformational changes (22, 23). This leads to binding of different antigens, but with different affinities (in our case, higher for AcMPAG than MPA). The presence of MPA in the samples did not attenuate the cross-reactivity of AcMPAG with the Emit antibody. Furthermore, interpretation of Emit test results must consider that AcMPAG, as a metabolite of MPA, shows a different pharmacokinetic profile compared with the parent drug and reaches its peak concentration \(c_{\text{max}} \) 1–3 h after the \(c_{\text{max}} \) of MPA (13). Therefore, the impact of AcMPAG on the MPA concentration determined with the Emit is relatively low during the first hour after MMF administration but increases considerably 4–8 h post dose (13). Fig. 1 shows a case in which AcMPAG reaches a concentration (1.85 mg/L) comparable to that of MPA (1.25 mg/L) 6 h after the MMF dose. In addition, the interindividual variation in MPA pharmacokinetics (1, 14) is also a factor that influences the AcMPAG/MPA ratio, which should be kept in mind when interpreting clinical data. Therefore, the role of Emit for the monitoring of MPA therapy with respect to AcMPAG needs further investigation with larger numbers of patients.

Another problem, which must be considered when analyzing biological specimens that contain acyl glucuronide conjugates of drugs, is the limited stability of these molecules. The stability depends on many factors, such as pH, temperature, and the nature of the aglycon (15). Our experiments with AcMPAG showed that rapid hydrolysis of this metabolite occurs when plasma is stored under physiological pH at room temperature as well as at 4 or \(-20^\circ\text{C}\). Only acidification to pH 2.5 immediately after collection of the samples, followed by storage at \(-20^\circ\text{C}\), provides satisfactory stabilization of AcMPAG for longer time periods (up to 1 month). Furthermore, the acidified samples were found to be stable at room temperature, which is of importance with respect to the handling and assay procedures. Concomitant loss of AcMPAG because of hydrolysis may lead to falsely increased MPA concentrations in plasma samples. This can be of importance, particularly when samples obtained 4–8 h after MMF intake are analyzed, because a major influence on the magnitude of this error will certainly be the ratio between MPA and AcMPAG concentrations in the plasma. Our results reconfirm findings with other acyl glucuronide metabolites (8, 9, 15) and demonstrate that careful collection, storage, and handling procedures should be used during the analysis of both AcMPAG and MPA to ensure the accurate collection of pharmacokinetic and toxicologic data when studies are performed with the immunosuppressant MPA.

In conclusion, the data presented here provide an analytical means to determine the AcMPAG concentrations in plasma and may allow investigation into their relevance with respect to both the immunosuppression and side effects related to MMF therapy.

Dr. M. Shipkova was supported by a grant from Volkswagen. We thank Christina Wiese and Tanja Schneider for excellent technical assistance. We also thank Drs. B. Tönshoff and L. Weber (University Hospital, Heidelberg, Germany) for the samples from pediatric renal transplant recipients.

References

10. Shipkova M, Niedmann PD, Armstrong VW, Schütz E, Wieland E, Oellerich M. Simultaneous determination of mycophenolic acid and its glucuronide in human plasma using a simple high-


