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Quantification of Mycophenolic Acid in Plasma Samples Collected during and Immediately after Intravenous Administration of Mycophenolate Mofetil, *Maria Shipkova,[1*] Victor William Armstrong,[1] Michael G. Kiehl,[2] Paul Dieter Niedmann,[1] Ekkehard Schütz,[1] Michael Oellerich,[1] and Eberhard Wieland[1]* ([1] Department of Clinical Chemistry, Georg-August-University Göttingen, D-37075 Göttingen, Germany; [2] Department of Hematology/Oncology, BMT Unit, Idar-Oberstein, Germany; * address correspondence to this author at: Abteilung Klinische Chemie, Zentrum Innere Medizin, Georg-August-Universität, Robert Koch Strasse 40, D-37075 Göttingen, Germany; fax 49-551-3912503, e-mail maria.shipkova@med.uni-goettingen.de)

Mycophenolate mofetil [(MMF); CellCept®; Roche Pharmaceuticals, Inc.], a prodrug of the immunosuppressive agent mycophenolic acid (MPA), is used for the prevention of rejection after organ transplantation. It is also under investigation for therapy of several autoimmune diseases, as well as prophylaxis for graft-vs-host disease in hematopoietic stem cell transplantation *(1, 2)*. After administration, MMF undergoes rapid and complete hydrolysis to MPA, its immunosuppressive active metabolite. The monitoring of plasma MPA is an important part of optimizing therapy with regard to pharmacologic and toxicologic effects *(3, 4)*. In addition to the commonly used oral MMF formulations, an intravenous formulation has been approved recently for prophylaxis against organ rejection in adult patients receiving allogenic renal or heart transplants. This intravenous solution enables MMF to be administered to patients unable to tolerate oral medication. The intravenous CellCept is given as an infusion of 1–3 h duration *(5)*. In spite of its rapid hydrolysis, the prodrug is potentially present in the plasma during and immediately after intravenous administration. Because MMF is very unstable and was found to undergo temperature-dependent degradation to produce MPA in human blood and plasma *(6)*, its presence in patient samples may critically affect the accuracy and precision of the analysis of MPA concentrations if these have to be measured for pharmacokinetic investigations during intravenous administration. The use of the Emit procedure with MMF-containing samples to monitor MPA is not possible because MMF cross-reacts with the antibody used in this assay *(7)*. However, most investigators are unaware of the potential analytic inaccuracy caused by MMF hydrolysis in samples containing this prodrug.

Ongoing multicenter trials with both bone marrow and heart transplant recipients require MPA monitoring during MMF infusion therapy to establish therapeutic efficiency and therapeutic ranges for this route of application. According to the study protocols, MPA concentrations have to be monitored during the first 1–3 h while the MMF infusion is still occurring *(5)*. In the study of Pescovitz et al. *(5)*, for example, patients received a 2-h intravenous infusion of MMF. Seven blood samples (at 20, 30, 40, 60, 80, 100, and 120 min) were taken during this infusion. Because we observed that samples sent to our laboratory from such trials frequently contained MMF, we reasoned that unpredictable hydrolysis of the prodrug may compromise the actual measured MPA plasma concentration. In our studies, we gave MMF as a 2- or 3-h infusion using the distal lumen of a central line. Samples from bone marrow transplant recipients were drawn by use of either the proximal lumen of the central line dedicated to drawing blood samples or venipuncture of a peripheral vein. MMF, as well as other infusions, was stopped, and a volume of 10 mL was aspirated and discharged. Immediately afterward, 5 mL of blood was collected and prepared for analysis. Blood from heart transplant recipients was obtained by venipuncture from the opposite limb not used for intravenous MMF infusion.

Tsina et al. *(6)* carefully investigated the stability of MMF to evaluate an HPLC method for its determination in human plasma and recommended specific precautions (immediate storage on ice, rapid processing, storage of plasma at −80 °C) to allow reliable MMF measurement. However, they neither discussed the significance of the MMF hydrolysis in vitro for the quantification of MPA nor did they investigate this phenomenon. Because the use of MMF as an intravenous formulation is becoming more common and the issue of whether AUC monitoring at time points during or shortly after intravenous application of MMF has not been settled, the importance of such an investigation is evident. We therefore evaluated the accuracy of MPA analysis in plasma samples containing MMF and also investigated possibilities to stabilize these samples.

For the present investigations, MMF, MPA, and the internal standard [carboxybutoxy ether of MPA (MPAC)] were a gift from Hoffmann-La Roche (Grenzach-Wyhlen, Germany). Sodium tungstate dihydrate and potassium dihydrogen phosphate, sodium hydroxide, phosphoric acid, and perchloric acid were from Merck. Acetonitrile (HPLC grade) was obtained from S. T. Baker B.V. Stock solutions of MPA and MPAC in acetonitrile, each at a concentration of 1 g/L, were prepared separately and stored at −20 °C. A 1 g/L stock solution of MMF was

prepared in acetonitrile–3 g/L phosphoric acid (90:10 by volume) to yield pH 2 and stored at −80 °C up to 1 month.

MPA and MMF were quantified according to an established procedure developed in our laboratory *(8, 9)*. Briefly, 200 μL of plasma and 100 μL of acetonitrile containing the internal standard MPAC (15 mg/L) were vortex-mixed 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.

The chromatographic separation was achieved with a Zorbax Eclipse XDB-C8 column [4.6 mm (i.d.) × 25 cm; Hewlett Packard] used as stationary phase. The mobile phase (flow rate, 1.2 mL/min) consisted of solution A (250 mL of acetonitrile and 750 mL of phosphate buffer, pH 3.0; final concentration, 20 mmol/L) and solution B (700 mL of acetonitrile and 300 mL of phosphate buffer, pH 6.5; final concentration, 20 mmol/L), which formed the following gradient: 0–4.5 min, 9% solution B; 4.5–9 min, 36% solution B; 9–13.5 min, 36% solution B; 13.5–14 min, 100% solution B; 14–17.5 min, 100% solution B; 17.5–18 min, 9% solution B. The column was maintained at 42 °C in a column temperature compartment (DuPont). The HPLC system consisted of a chromatographic pump (M480), an automatic injector (GINA 50), a diode array detector (UVD 340S), and a computer interface system controller linked to a PC (Dionex-Gynkotek). The compounds were quantified by absorbance at 215 nm, using peak-area ratios. The calibration and the quality control were performed by use of in-house calibration solutions that were analyzed with each analytic run as described previously *(8, 9)*. This method is linear up to 50 mg/L for MPA and up to 100 mg/L for MMF. Detection limits are 0.01 and 0.02 mg/L for MPA and MMF, respectively. The recoveries are 99–103% for MPA (working range, 0.2–25 mg/L) and 94–102% for MMF (working range, 0.2–100 mg/L). The within-day imprecision (CV) is <5.0% for MPA and <4.1% for MMF, and the between-day imprecision is <6.2% and <8.1%, respectively.

To investigate the influence of MMF hydrolysis in vitro on MPA concentrations, we started our study with an experiment in which we pooled plasma samples obtained from a bone marrow recipient receiving intravenous therapy with MMF (1 g of MMF twice a day). In one aliquot of this pool, MPA and MMF concentrations were immediately analyzed, and a further three aliquots were measured after 1, 3, and 6 h of storage at room temperature, respectively. In addition, plasma samples containing MMF from 12 patients on intravenous MMF treatment were stored for 1 week at 4 °C. MPA concentrations determined in these samples before and after storage were compared, and the critical difference (d_k) was calculated *(10)*. The d_ks were obtained according to the formula $d_k = 2 \times \sqrt{2} \times s$, where s represents the SD 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 ($|x_1 - x_2| > d_k$) *(10)*. All analyses were performed in duplicate.

Storage of the plasma pool at room temperature led to an increase in the MPA concentration from 4.62 mg/L to 8.01 mg/L (173.4%) after 1 h, to 12.19 mg/L (263.9%) after 3 h, and to 16.25 mg/L (351.7%) after 6 h. This increase was paralleled by a comparable decrease in the MMF concentration. Storage of patient samples (n = 12) at 4 °C for 1 week, which were collected during intravenous therapy and contained MMF, also led to a substantial increase of the MPA concentrations in all samples. The initial median values were 4.64 mg/L for MPA (range, 1.84–29.43 mg/L) and 6.94 mg/L for MMF (range, 2.37–57.14 mg/L). After 1 week at 4 °C, the corresponding median values were 9.12 mg/L for MPA (range, 2.47–79.11 mg/L) and 1.70 for MMF (range, 0.81–9.5 mg/L). A very good correlation was found between the MMF concentrations measured in the samples before storage and the increase of the MPA concentrations during the storage time (Spearman, $r = 0.94$). These results clearly demonstrate that MPA monitoring is compromised severely if in vitro hydrolysis of MMF is not prevented in samples from patients receiving intravenous CellCept, particularly when these samples are obtained during, or immediately after, infusion. Therefore, we performed further experiments aimed at developing specific storage and processing conditions, which could guarantee accurate measurement of MPA.

Two different plasma pools from patients not on MMF therapy were supplemented with 50 mg/L MMF. This concentration was chosen because of our experience showing that MMF concentrations up to 60 mg/L are found in plasma from patients during the infusion period. An aliquot from each pool was acidified with phosphoric acid (850 g/L) to yield a pH of ~2.5 (e.g., 10 μL of phosphoric acid/500 μL of plasma). At this pH, ester bonds are known to be fairly resistant to hydrolysis *(11)*. In addition, we have shown previously that hydrolysis of the acyl glucuronide of MPA can be efficiently prevented by this treatment *(9)*. The acidified pools were stored at either 4 °C or −20 °C *(8)* in 200-mL aliquots; the nonacidified pools were stored separately at 4 °C or −80 °C *(6)*.

We determined MPA concentrations immediately after preparation of the pools and after 1 day, 3 days, 7 days, and 30 days of storage. We performed all analyses in duplicate and compared the values by calculating the critical differences (d_ks) as described above.

In vitro MPA formation from MMF during storage under different conditions is shown in Fig. 1. Although different pools were investigated to study the effect of different matrices, the pattern of MPA formation was almost identical. The initial values in the nonacidified samples were already increased compared with the acidified samples because of the time elapsed during pool preparation and aliquoting. Because this was performed at room temperature, MMF degradation will have occurred in samples at physiologic pH as discussed above. Whereas the MPA concentration steadily increased over the 30 days in the nonacidified samples kept at 4 °C, this

was largely prevented at pH 2.5. At this pH, MPA concentrations did not significantly change after 3 days at 4 °C. However, after 7 days, MPA values were approximately doubled compared with the initial values and reached four- to fivefold concentrations after 30 days.

As reported previously by Tsina et al. *(6)*, hydrolysis of MMF can be prevented by storage at −80 °C. However, as shown in Fig. 1, there is considerable variation in MPA values determined after thawing of such samples. This is most likely attributable to the variable and unpredictable conversion of MMF to MPA during sample preparation because hydrolysis is not prevented at the temperatures and pH values present after thawing of the plasma. In contrast, hydrolysis of MMF was completely prevented over the storage time and during sample preparation after thawing at the conditions (−20 °C, pH 2.5) previously shown to be suitable to stabilize the acyl glucuronide of MPA *(9)*. When acidified MMF-containing samples were thawed and allowed to stand for 6 h at room temperature, the MPA concentrations determined hourly did not significantly increase over time.

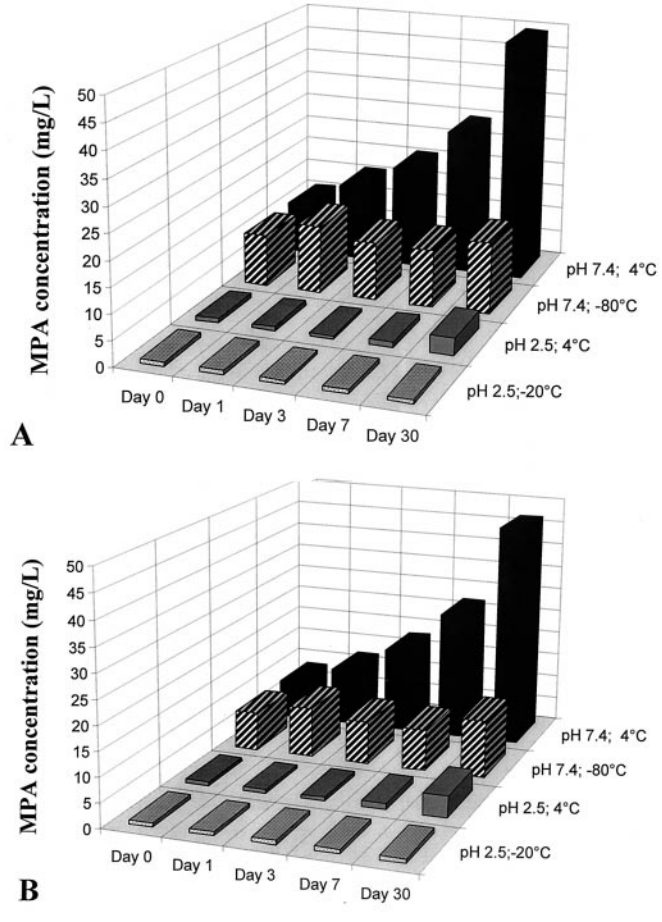

Fig. 1. Overall changes in the concentrations of MPA in acidified and nonacidified plasma samples stored for up to 30 days at 4 °C, −20 °C, or −80 °C.

The experiment was performed in parallel with two different plasma pools (*A* and *B*). All data are given as mean from duplicate analyses.

These results show that MPA monitoring in MMF-containing samples is critically affected by hydrolysis of the prodrug during storage and sample preparation. The most effective means to prevent this hydrolysis for longer time periods is acidification of the samples and storage at temperatures of −20 °C or lower. In addition, the low pH used during sample pretreatment in our HPLC method is favorable with respect to the stability of MMF and allows analysis of large series with an autosampler. Because acidification of whole blood will prevent other determinations from the same sample, we have not followed this approach. Instead, plasma and cells were separated as soon as possible after blood collection. To prevent MMF degradation before centrifugation, samples can be stored on ice for up to 30 min. In 10 whole-blood samples containing 2.40 ± 0.57 mg/L MPA and 11.45 ± 2.20 mg/L MMF, respectively, 2.37 ± 0.49 mg/L MPA and 11.47 ± 2.18 mg/L MMF were found after 30 min of storage on ice ($P > 0.05$, paired t-test). Separation of plasma and cells was performed in a refrigerated centrifuge at 0 °C.

Because MMF has no immunosuppressive activity, determination of MPA is required if monitoring of therapy is performed for transplant recipients. In this case, careful collection, storage, and handling procedures should be used, particularly when the samples are obtained during or immediately after infusion. This is especially important for pharmacokinetic investigations because in vitro hydrolysis of MMF could lead to an overestimation of the calculated MPA areas under the curve. If measures are not taken to prevent the conversion of MMF to MPA in vitro (i.e., during separation, handling, and storage of the plasma sample), the measured plasma MPA concentration in vitro will be higher than the true plasma concentration in vivo. Furthermore, because the MMF present in the circulation at this particular sampling time point will be converted to MPA in vivo, the MMF concentration will contribute to the MPA plasma pool and, therefore, the MPA concentration at the next measurement time point. The net result will be an overestimation of the MPA area under the curve. In addition to the pharmacokinetic investigations, we have occasionally observed that MMF may be present in samples at later time points after infusion because of blood sampling from the infusion line used to administer MMF.

From our experience, sample stabilization according to the protocol outlined above has become mandatory in ongoing clinical trials with intravenous MMF, which sometimes require blood sampling during or immediately after infusion.

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Extracellular Tyrosinase mRNA within Apoptotic Bodies Is Protected from Degradation in Human Serum,

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The amplification of tyrosinase mRNA by reverse transcription-PCR (RT-PCR) from peripheral blood has frequently been used as a marker for the presence of circulating tumor cells in melanoma patients *(1)*. Recently, tyrosinase mRNA was also demonstrated to be detectable in serum and plasma samples from melanoma patients, indicating that tumor-specific mRNA is present in an extracellular form in the bloodstream *(2, 3)*. This has been confirmed by other investigators demonstrating the presence of extracellular telomerase mRNA in the serum of breast cancer patients *(4)*. It has been speculated that the presence of tumor-associated mRNA in serum and plasma is possibly related to tumor cell necrosis and release of transcripts into the circulation. Furthermore, there is evidence to suggest that mRNA may be actively secreted by tumor cells in vivo *(5, 6)*. It is not yet clear, however, how serum and plasma mRNA is protected from RNases. Several findings have indicated that extracellular RNA might be protected from serum RNases by proteins or proteolipid complexes *(6–8)*. Very recently, it was demonstrated that DNA and RNA are packaged separately into apoptotic bodies, producing two types of apoptotic bodies, one type containing RNA and no DNA and the other type containing DNA and no RNA *(9)*. This finding suggests that extracellular mRNA may circulate within apoptotic bodies, which may decrease their susceptibility to nucleases. The present study was therefore conducted to analyze the serum stability of tyrosinase mRNA associated with apoptotic bodies in comparison with free tyrosinase transcripts.

Cells (1×10^6 cells/well) of the melanoma cell line Bu-Hom (established in our laboratory from a patient's lymph node metastasis) were plated in 6-well tissue culture plates (Costar) in 2 mL of medium consisting of Dulbecco's modified Eagle's medium containing 100 mL/L fetal serum and incubated at 37 °C in 10% CO_2. Cells were allowed to adhere overnight. Apoptosis of melanoma cells was induced by the addition of the agonistic cross-linking anti-CD95 monoclonal antibody 7C11 (50 μg/L; Immunotech) for 48 h. At the termination of culture, cell-free supernatants were collected and passed through a 0.45 μm filter.

Induction of apoptotic cell death and apoptotic bodies was confirmed by different methods. In the first method, melanoma cells were precisely analyzed by light microscopy, and morphologic changes characterizing apoptotic cell death were identified. In the second method, apoptotic bodies present in collected cell-free supernatants were identified by double staining and subsequent flow cytometry using fluorescein isothiocyanate-labeled annexin V, a marker for early and late apoptosis, and propidium iodide, a marker for late apoptosis and necrosis (TACS Annexin V Apoptosis Detection reagent set; Genzyme). These studies confirmed the presence of apoptotic bodies in culture supernatants of melanoma cells incubated with monoclonal antibody 7C11. In the third method, apoptotic bodies present in supernatants were attached to microscope slides, fixed in ice-cold solution containing 10 g/L methanol-free formaldehyde in phosphate-buffered saline for 15 min, and postfixed in cold 700 mL/L ethanol for at least 2 h. The presence of RNA in apoptotic bodies was confirmed by staining with pyronin Y (20 mg/L; Sigma). Fluorescence microscopy (570 nm) identified typical granular structures representing apoptotic bodies that contained RNA.

Cell-free supernatant (1 mL) from 1×10^6 cultured melanoma cells was mixed with 2 mL of fresh serum from controls and incubated at 37 °C for different periods of time. As a control, 1 μg of free total RNA isolated from Bu-Hom melanoma cells was mixed with normal serum. After the indicated time points, serum samples were stabilized with RNA/DNA Stabilization Reagent for Blood/Bone Marrow (Roche Diagnostics). Poly(A)$^+$ mRNA was extracted using the mRNA Isolation Kit for Blood and Bone Marrow (Roche Diagnostics) according to the manufacturer's protocol. cDNA was synthesized and amplified in a 50-μL PCR reaction using the Qiagen One Step RT-PCR reagent set (Qiagen). In brief, 6 μL of mRNA was reverse-transcribed with specific primers for tyrosinase, using One Step RT-PCR enzyme mixture (Qiagen), and then amplified in the following PCR reaction using HotStarTaq DNA polymerase (Qiagen). The following tyrosinase primers were used in PCR reactions: 5′-TTGGCAGATTGTCTGTAGCC-3′ (sense) and 5′-AGGCATTGTGCATGCTGCTT-3′ (antisense). Cycling conditions were as follows: for reverse transcription of mRNA, 30