

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/12134593>

Determination of Mycophenolic Acid and Mycophenolate Mofetil by High-Performance Liquid Chromatography Using Postcolumn Derivatization

ARTICLE in ANALYTICAL CHEMISTRY · JANUARY 2001

Impact Factor: 5.64 · DOI: 10.1021/ac0006730 · Source: PubMed

CITATIONS

29

READS

60

5 AUTHORS, INCLUDING:



Ulf Renner

PharmaZell

20 PUBLICATIONS 406 CITATIONS

SEE PROFILE



Christian Thiede

Universitätsklinikum Dresden

280 PUBLICATIONS 10,206 CITATIONS

SEE PROFILE

Determination of Mycophenolic Acid and Mycophenolate Mofetil by High-Performance Liquid Chromatography Using Postcolumn Derivatization

Ulf D. Renner,^{*,†} Christian Thiede,[†] Martin Bornhäuser,[†] Gerhard Ehninger,[†] and Hans-Michael Thiede[‡]

Medizinische Klinik und Poliklinik I, Universitätsklinikum Carl Gustav Carus der Technischen Universität, Fetscherstrasse 74, D-01307 Dresden, Germany, and Analyticon AG, Hermannswerder, Haus 17, D-14473 Potsdam, Germany

An efficient method to lower the optical detection limit is described using the displacement of an absorption and emission band of an analyte after a polarity change in different solvents. This solvatochromic effect was used in a RP-HPLC assay for the fluorescence detection of mycophenolic acid (6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-5-phthalanyl)-4-methyl-4-hexenoic acid, MPA) and the prodrug mycophenolate mofetil (MMF), the *N*-(2-hydroxy-ethyl)morpholino ester of MPA. The rationale to use fluorescence detection is based on the behavior of MMF and MPA, which fluoresce in a basic medium (pH > 9.5). Following a simple protein precipitation, the analytes were separated in an isocratic RP-HPLC system. The postcolumn generation of the phenolate anions of MPA and MMF was achieved by addition of an aqueous sodium hydroxide solution regulated by a newly developed continuous-flow liquid control system. MPAG, not directly accessible for fluorescence detection, was analyzed after enzymatic deglucuronidation to MPA. Compared to published quantification limits for MPA and MMF by UV detection, this method is more than 100-fold more sensitive, with a lower limit of quantification of 45 fmol for both MPA and MMF.

Highly sensitive assays for an in vivo analysis of drugs in different physiological compartments are increasingly demanded to reveal biochemical interactions and pharmacological effects on cells in different matrixes (e.g., the metabolism in tumors). Frequently, HPLC methods using UV detection lack the necessary sensitivity and are thus not suitable for determination of drugs and its metabolites in biological matrixes directly. To achieve sufficient sensitivity, extensive preanalytical preparation (e.g., solid-phase extraction) has to be performed frequently in order to enrich the substances of interest. These procedures are time-consuming and expensive.

We describe a simple method to improve the limit of detection for an analyte having a suitable molecular structure to create a change of the photophysics in different solvents. The solvatochromic effect frequently described in dye chemistry (e.g., phthalein and triphenylmethane derivatives (pH indicators), mero-

cyanine, *N*-phenolpyridinium betaine) was used for the development of a reversed-phase HPLC assay for mycophenolic acid (MPA) and mycophenolate mofetil (MMF).

MMF was developed as a prodrug of the antibiotic MPA, which is produced by several species of the genus *Penicillium*. MPA has been shown to be an efficient drug for the prevention of acute rejection in patients receiving organ transplants.^{1–7} MMF is rapidly and completely absorbed and hydrolyzed to MPA after oral application. For a short time during and following an intravenous administration, the rapid conversion of MMF to MPA can be detected in plasma. The drug exerts its immunosuppressive activity only after hydrolysis to MPA. The immunosuppressive effects are based on the reversible, noncompetitive inhibition of inosine monophosphate dehydrogenase (IMPDH). This enzyme is involved in the de novo synthesis of the purine nucleotide, guanosine monophosphate. Due to this pharmacologic action of MPA, the intracellular guanine nucleotide pool decreases significantly, which results in an inhibition of DNA synthesis in a MPA dose-dependent manner.^{8,9} Lymphocytes rely on de novo purine biosynthesis more than the purine salvage pathway (catalyzed by hypoxanthine-guanine phosphoribosyl transferase). Because of this dependence, treatment with MPA preferentially inhibits the proliferation and functions of the T- and B-lymphocytes.

Our assay allows direct analysis of MPA and MMF in different biological fluids. The glucuronide MPAG was determined after enzymatic conversion to the aglycon MPA (Figure 1). MPAG is a pharmacologically inactive metabolite but is hydrolyzed in vivo to MPA. Since MPAG is excreted in bile, the enterohepatic recirculation contributes to plasma concentration of MPA shown

- (1) Sollinger, H. W. U.S. Renal Transplant Mycophenolate Mofetil Study Group. *Transplantation* **1995**, *60*, 225–232.
- (2) The European Mycophenolate Mofetil Cooperative Study Group. *Lancet* **1995**, *345*, 1321–1325.
- (3) Bullingham, R. E. S.; Nicholls, A.; Hale, M. *Transplant. Proc.* **1996**, *28*, 925–929.
- (4) Langman, L. J.; LeGat, D. F.; Halloran, P. F.; Yatscoff, R. W. *Transplantation* **1996**, *62*, 666–672.
- (5) Wolfe, E. J.; Mathur, V.; Tomlanovich, S.; Jung, D.; Wong, R.; Griffy, K.; Aweeka, F. T. *Pharmacotherapy* **1997**, *17*, 591–598.
- (6) Nicholls, A. J. *Clin. Biochem.* **1998**, *31*, 329–333.
- (7) Hale, M. D.; Nicholls, A. J.; Bullingham, R. E. S.; Hené, R.; Hoitsma, A.; Squifflet, J.-P.; Weimar, W.; Vanrenterghem, Y.; Van de Woude, F. J.; Verpooten, G. A. *Clin. Pharmacol., Ther.* **1998**, *64*, 672–683.
- (8) Eugui, E. M.; Almquist, S. J.; Muller, C. D.; Allison, A. C. *Scand. J. Immunol.* **1991**, *33*, 161–173.
- (9) Sintchak, M. D.; Fleming, M. A.; Futer, O.; Raybuck, S. A.; Chambers, S. P.; Caron, P. R.; Murcko, M. A.; Wilson, K. P. *Cell* **1996**, *85*, 921–930.

* Corresponding author: (phone) -49-351-458-4672; (fax) -49-351-458-5362; (e-mail) renner@oncocenter.de.

[†] Universitätsklinikum Carl Gustav Carus der Technischen Universität.

[‡] Analyticon AG.

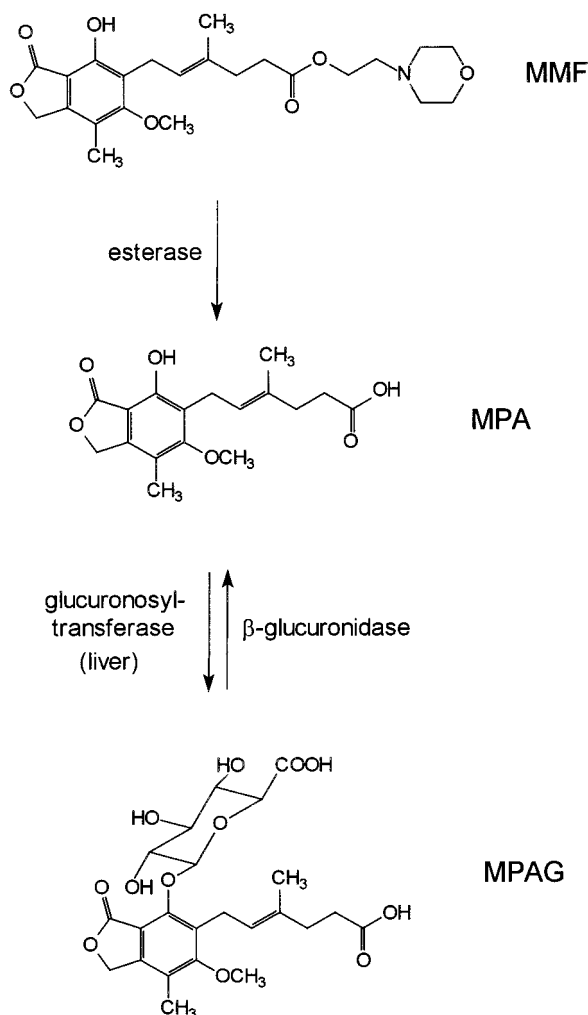


Figure 1. Structures of the prodrug mycophenolate mofetil (MMF), mycophenolic acid (MPA), and the metabolite mycophenolic acid glucuronide (MPAG) and their enzymatically catalyzed transformation.

in the plasma concentration versus time profile by a secondary peak plasma MPA concentration 6–12 h after administration.¹⁰ To determine concentrations of the drug in plasma or in urine, we used an isocratic HPLC system applying fluorescence detection. The principal basis for the detection method is the behavior of MPA and MMF, which show a strong fluorescence in a basic environment (pH 10). Using this solvatochromic reaction, the eluent system was changed on-line by addition of a 2 N sodium hydroxide solution after the chromatographic separation. The amount of the sodium hydroxide added was regulated by a newly developed system using pulsation-free gas pressure and electronic regulation by means of a liquid flow controller.

EXPERIMENTAL SECTION

Reagents. Acetone (99.9+%, HPLC grade) and tetrahydrofuran (99.9+%, HPLC grade) were purchased from Sigma-Aldrich (Steinheim, Germany). Methanol (Chromasolv, HPLC grade) was obtained from Riedel-deHaën (Seelze, Germany). Acetonitrile (HPLC grade) and acetic acid (99+%) were obtained from J. T. Baker (Deventer, Netherlands). The internal standard, (*S*)-6-

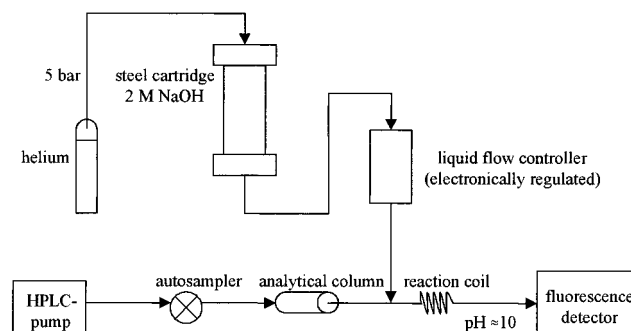


Figure 2. Arrangement of the HPLC equipment connected to the postcolumn derivatization unit.

methoxy- α -methyl-2-naphthaleneacetic acid ((+)-naproxene), and mycophenolic acid ($C_{17}H_{20}O_6$; FW 320.34; 98% purum) were obtained from Sigma (Deisenhofen, Germany); 2-(4-morpholino)-ethyl-(*E*)-6-(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuran-4-methyl-4-hexenoate ($C_{23}H_{31}NO_7$; FW 433.50) was obtained from Roche (Basel, Switzerland); and ethylenediamine-(*N,N,N,N*)-tetraacetic acid disodium salt (EDTA) was from Aldrich (Steinheim, Germany). Citric acid monohydrate, ammonium acetate, and sodium hydroxide were purchased from Merck (Darmstadt, Germany). The enzymes β -glucuronidase/arylsulfatase from *Helix pomatia* (stored at +4 °C) were obtained from Boehringer Mannheim (Mannheim, Germany). HPLC grade, deionized water was generated using a Milli-Q-Plus PF water purification system (Millipore, Eschborn, Germany) for preparations of HPLC mobile-phase and sample solutions.

Instrumentation. Chromatography was performed using a Thermo-Separation-Products Spectra System (Thermo Quest, Eggenstein, Germany). The system consisted of a SCM 1000 vacuum membrane degasser, a P2000 HPLC pump, an AS3500 autosampler equipped with a 100- μ L sample loop (PEEK material, Thermo Quest), and a FL3000 fluorescence detector set to an excitation wavelength of 340 nm and an emission wavelength of 450 nm. Acquired data were processed by the PC1000 software (Thermo Quest). Separations were performed on an Spherisorb ODS-2, (5 μ m) HPLC cartridges (60 \times 4 mm, Waters, Eschborn, Germany), protected with an Spherisorb ODS-2 (10 \times 4 mm, 5 μ m) C-18 guard column.

For postcolumn deprotonation of MMF and MPA, a 2 M aqueous sodium hydroxide solution was added to the isocratic eluent system resulting in a pH value of 10. The tee connection (PEEK mixing TEE, Thermo Quest) was located between HPLC column and the fluorescence detector (Figure 2). Following the tee connection, a capillary coil (diameter 0.3 mm, length 40 cm; Knauer, Berlin, Germany) was installed for the deprotonation reaction and for homogenizing the reaction mixture.

The equipment for postcolumn derivatization consisted of a stainless steel cartridge (40 \times 250 mm; Knauer) filled with 2 M sodium hydroxide solution. One end of the cartridge was connected to an helium gas cylinder (Helium 5.0; Messer, Griesheim, Germany), applying a constant pressure (0.5 MPa) to the NaOH solution in the steel cartridge. The exit of the stainless steel cartridge was connected to a Bronkhorst liquid flow controller (Mättig, Unna, Germany) using stainless steel tubing. The electronic regulation of this valve was carried out by a Bronkhorst

(10) Lee, W. A.; Gu, L.; Mikszta, A. R.; Chu, N.; Leung, K.; Nelson, P. H. *Pharm. Res.* **1990**, *7*, 161–166.

HI-TEC Flow Bus (Mättig) and was independent of the HPLC system. The flow rate added to the eluent system was 2 mL of 2 M NaOH/h.

Chromatographic Conditions. The analysis was performed using an isocratic eluent system. The solvent system contained an aqueous buffer solution, methanol, tetrahydrofuran, and acetone. One liter of the aqueous buffer solution (pH 4.5) consisted of 5.25 g of citric acid, 1.4 g of sodium hydroxide, and 0.5 mL of an EDTA solution (7.44 g of EDTA disodium salt in 100 mL of deionized water). Two hundred milliliters of methanol, 135 mL of tetrahydrofuran, and 90 mL of acetone were mixed and filled up to 1 L with the filtered (PTFE, 0.5 μ m; diameter 47 mm; Millipore) aqueous buffer solution. The flow rate was maintained at 0.5 mL/min.

In the following parts of the Experimental Section, the procedure for drug analysis in plasma is described. Because the sample preparation of the HPLC assay is independent of biological medium containing the MPA and MMF, the extraction procedure can also be applied to urine samples.

Standards. Stock solutions of MPA (100.0 μ g/mL) and MMF (50.0 μ g/mL) in acetone were stable at +4 °C for more than 4 weeks and were further diluted with water prior to use. The diluted MPA solutions of 0.156–50.0 μ g/mL were stored at +4 °C for 4 weeks; however, the MMF solutions (in a range of 0.78–50.0 μ g/mL) were prepared freshly. For preparation of a plasma calibration curve, 20 μ L of the MPA or MMF spiking solutions was added to 980 μ L of blank human plasma in order to receive the corresponding calibration standards. To minimize the ester hydrolysis of MMF caused by enzymatic activity present in plasma, aliquots of the MMF spiking solutions were always diluted with cooled plasma (10 °C). After vortexing (10 s), the enzymatic activity was immediately stopped by addition of 400 μ L of solvent mixture to 0.1 mL of the spiked calibration plasma sample in order to precipitate the plasma proteins. The solvent mixture contained (*S*)-6-methoxy- α -methyl-2-naphthaleneacetic acid (internal standard, 125.0 μ g/mL) dissolved in methanol and acetonitrile in a ratio of 40:60 (v/v). In the organic solvent system the (*S*)-6-methoxy- α -methyl-2-naphthaleneacetic acid was stable at +4 °C for more than 2 weeks. Further processing of the calibration plasma samples is described in the following section.

Sample Preparation. Samples of potassium EDTA plasma were obtained from healthy volunteers or from patients receiving MMF intravenously or orally. After venous blood was collected, the sample was immediately centrifuged for 10 min at 10 °C (1500 rpm; Hettich Rotina 48 R, Tuttlingen, Germany). Aliquots of plasma were frozen and stored at –80 °C until analysis. Under these storage conditions, MPA and especially MMF in plasma were found to be stable for more than 3 months (data not shown).

Extraction of Clinical Plasma Samples: Determination of MPA and MMF. The plasma samples were thawed and vortexed for 10 s just before the extraction of the plasma was carried out. Vials containing the plasma samples were not allowed to reach a temperature of more than 10 °C in order to inhibit a significant decomposition of MMF by enzymatically catalyzed hydrolysis. For protein precipitation, 0.1 mL of the plasma sample was diluted with 0.4 mL of the methanol/acetonitrile solvent mixture containing the internal standard. The rest of thawed plasma was cooled in an ice/water bath immediately. The organic solvent/plasma

mixture was vortexed for 45 s and centrifuged in a benchtop microcentrifuge at 15 000 rpm and ambient temperature. The upper layer was completely removed and added to 400 μ L of deionized water. After vortexing, 40 μ L of the mixture was injected into the HPLC system.

Extraction of Clinical Plasma Samples: Determination of MPAG. Twenty microliters of the thawed plasma was added to 80 μ L of an enzyme/buffer mixture (20 mL of this enzyme/buffer solution contained 1 mL of a 2 M ammonium acetate buffer (pH 6.5) and 100 μ L of β -glucuronidase/arylsulfatase solution). The mixture was incubated at 37 °C in a water bath and shaken for 1 h. To stop the enzymatic hydrolysis reaction, plasma proteins were precipitated as described above. The supernatant was completely removed and further diluted with deionized water to yield an absolute volume of 1.5 mL. A 25- μ L aliquot of this dilution were injected into the HPLC system.

Assay Validation. Plasma calibration curves were prepared, each consisting of one blank plus seven samples containing MPA and MMF, respectively. To evaluate the linearity and the precision (intraassay and interassay), spiked plasma samples were analyzed in quadruplicate on 5 days. The recovery was analyzed by comparison of peak areas for MPA and MMF in plasma to equivalent amounts of the drug and prodrug dissolved in the isocratic eluent and injected directly into the HPLC system. The recoveries of MPA and MMF were evaluated at MPA and MMF concentrations of 1000.0, 200.0, 100.0, 50.0, 25.0, 12.5, 6.25, and 3.125 ng/mL and 1000.0, 500.0, 250.0, 125.0, 62.5, 31.25, and 15.62 ng/mL plasma, respectively. The limit of detection was determined by analysis of plasma samples containing 3.00, 6.00, and 100.0 ng/mL mycophenolic acid and mycophenolate mofetil.

Calculation of MPAG Concentration in Plasma. Under the described HPLC conditions, MPAG is undetectable using fluorescence detection, since the phenolic function in the molecular structure is protected by a glucuronyl substituent. Therefore, we determined the plasma levels of MPAG as equivalents of MPA after an enzymatic deglucuronidation reaction. The hydrolysis of the glycosidic bond results in the aglycon MPA. The increased amount of MPA was analyzed by an additional HPLC run after enzymatic deglucuronidation. The following equation was used for the calculation of the MPAG plasma levels,

$$\text{concn}_{\text{MPAG, plasma}} = B[\text{concn}_{\text{MPA, after enzyme reaction}} - A(\text{concn}_{\text{MMF, before enzyme reaction}} - \text{concn}_{\text{MMF, after enzyme reaction}})]$$

where *A* is $\text{FW}_{\text{MPA}}/\text{FW}_{\text{MMF}}$ and *B* is $\text{FW}_{\text{MPAG}}/\text{FW}_{\text{MPA}}$ (FW, formula weight).

RESULTS AND DISCUSSION

Theoretical Background. The principal idea of the described method is to use the solvatochromic effect for the detection of MPA and MMF. We reasoned that the basic condition generated by addition of 2 M NaOH to the solvent system leads to the almost complete dissociation of the acidic proton of the unprotected phenol function of MPA and MMF just before fluorescence detection. The resulting phenolate anion lowers the difference of the energy between the electronic ground state of the molecule and the excited states. The upward and downward electronic transitions, respectively, correspond to frequencies of visible and

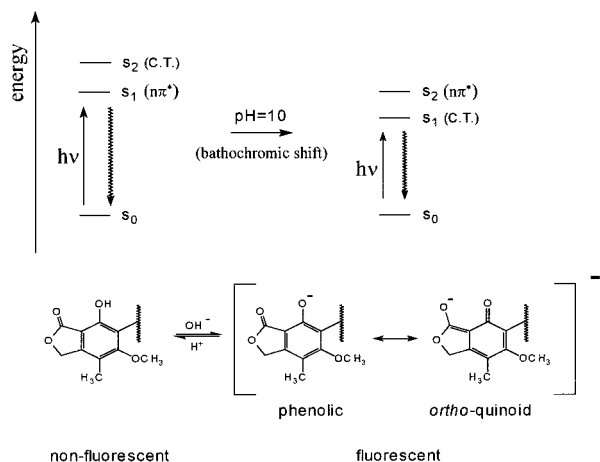


Figure 3. Energy levels of MPA and deprotonated MPA in increasing polar and protic solvent systems.

near-UV light (bathochromic shift). Following the change of the polarity of MPA, the electron distribution is combined with a different permanent dipole moment (before/after solvent change) and a crossing of two excited electronic states. In polar solvents, electronic states of large dipole moments (e.g., charge-transfer (CT) states) are stabilized with respect to states of small dipole moments. When these states are close in energy, an inversion of this order can take place, when the polarity of the solvent is changed (Figure 3). Since the photophysical behavior of molecules is determined by the lowest excited state of a given spin quantum number, these properties can show very large variations with solvent polarity.

The basicity of deprotonated MPA is strongly influenced by its electrostatic charge distribution. The partial negative charge δ^- of the oxygen atom of the phenolate group determines the electrostatic energy of this oxygen atom. In the electronic ground state, the phenolate function is strongly basic with three non-bonding electron pairs located at the oxygen atom. In the lowest singlet excited electronic state, the deprotonated hydroxy function becomes a weak base. A charge transfer from the O atom of the phenolic group to the phthalanyl ring system takes place, which lowers the density of electrons at the oxygen atom. Additionally the electrostatic attraction for a proton decreases as well. In the ground state, the π -electrons of the phthalanyl system are in conjugation with a p-orbital of the adjacent oxygen atom of the phenolate group (delocalized π -electrons; resonance energy).

The interactions between a solute and the surrounding solvent molecules of a liquid can be described as nonspecific (e.g., solute dipole–solvent dipole, solute dipole–solvent polarization) and specific electrostatic interactions. A neutral molecule (solute and solvent) can be defined through its permanent dipole moment $\vec{\mu} = \alpha \vec{E}$ (molecule polarizability α in direction of the external electric field \vec{E}). The total polarization of a solvent considered as a continuum is described by the Onsager polarity function $f(D) = 2(D - 1)/(2D + 1)$ and is characterized by the orientation polarization ($f(D) - f(n^2)$), dependent on the macroscopic dielectric solvent constant D and the square of the refractive index n and the induction polarization ($f(n^2)$), dependent on n^2 as a function of

the solvent polarizability α_s).¹¹ In the specific solute–solvent associations, the decisive interactions are mediated by hydrogen bonding. Such interactions enable important effects on photo-physics of solute molecules, e.g., a solvatochromic shift.

Following the displacement of the absorption and emission bands, a linear increase of the transition energy $E = h\nu$ within a differential polarity increment is observed (hypsochromic shift), if the dipole moment of the excited molecule is smaller than that of the ground state (e.g., $n\pi^*$ state of a carbonyl compound). In the reverse case, the gradient slope becomes negative (bathochromic shift), if the dipole moment increases in the excited electronic state of the solute (e.g., CT state).

However, on the basis of a dielectric enrichment, the solvatochromic reaction of the phenolate anions is much more complex. Since the solvent mixture shows different polarities for each solvent ($f(D_1)$, $f(D_2)$, ..., the Onsager polarity function has to be modified, depending on the mole fractions. If the solute has an ionic character, the Onsager function has to be replaced by the Born equation $F(D) = (1 - 1/D)$. However, the theoretical model developed by Born is based on a spherical ion with a concentric single charge without a dipole moment.¹² Up to now, there is no theory about the solvatochromic shift of ionic molecules.

Experimental Results. A representative chromatogram obtained from the analysis of a plasma sample from a patient receiving MMF at 28 mg/kg body weight (intravenous administration) is shown in Figure 4 compared to chromatograms from blank human plasma and a spiked plasma sample. The retention times of MMF, MPA, and (+)-naproxene (internal standard) were 6.0, 8.9, and 11.5 min, respectively.

(+)-Naproxene did not interfere with the MPA or MMF, and the use of this internal standard enabled compensation for any sample-to-sample variations in the extraction procedure.

Using the extreme solvatochromic reaction of the analytes, very low limits of detection for MPA and MMF were obtained with the described HPLC assay. To analyze the drug, an increase in basicity ($\text{pH} > 10$) does not improve the fluorescence intensity of the analyte. The determination of yields of fluorescence of an analyte solution adjusted by different pH values showed a maximum fluorescence intensity at pH 10 (data not shown). When 100 μL of plasma was extracted, the lower limits of detection (signal-to-noise ratio (S/N) 3:1) were 45 fmol for MPA (15 pg) and MMF (19.5 pg). With regard to the described sample preparation, these amounts correspond to plasma concentrations of 3.3 and 4.4 ng/mL for MPA and MMF, respectively. This is an improvement of ~ 2 orders of magnitude compared to published HPLC methods investigating the pharmacokinetics of MPA, MPAG, and MMF in human plasma.^{13–22} In these assays, UV detection (254 nm) is used and the lower quantification limits for

- (11) Atkins, P. W. *Physical Chemistry*, 3rd ed.; Oxford University Press: New York, 1986.
- (12) Suppan, P. *Chemistry and Light*, 1st ed.; The Royal Society of Chemistry: Cambridge, 1994.
- (13) Sugioka, N.; Odani, H.; Ohta, T.; Kishimoto, H.; Yasumura, T.; Takada, K. *J. Chromatogr., B* **1994**, 654, 249–256.
- (14) Shaw, L. M.; Nowak, I. *Ther. Drug Monit.* **1995**, 17, 685–689.
- (15) Tsina, I.; Kaloostian, M.; Lee, R.; Tarnowski, T.; Wong, B. *J. Chromatogr., B* **1996**, 681, 347–353.
- (16) Li, S.; Yatscoff, R. W. *Transplant. Proc.* **1996**, 28, 938–940.
- (17) Huang, J. J.; Kiang, H.; Tarnowski, T. L. *J. Chromatogr., B* **1997**, 698, 293–300.
- (18) Saunders, D. A. *J. Chromatogr., B* **1997**, 704, 379–382.

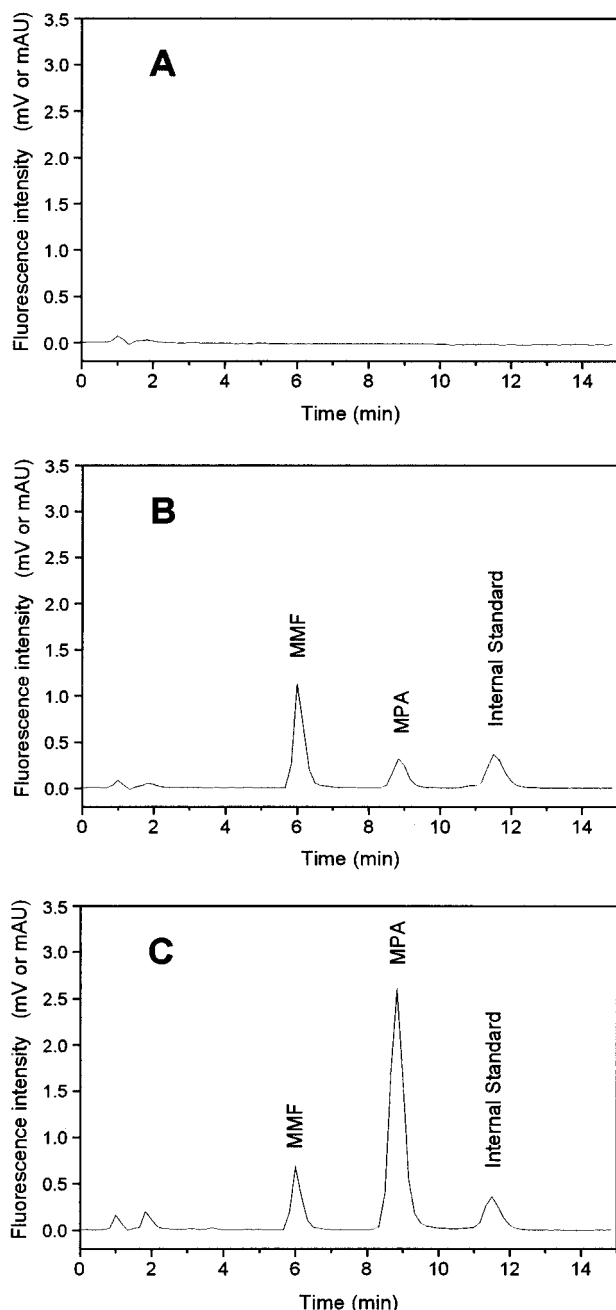


Figure 4. Typical chromatograms (fluorescence detection) of (A) blank plasma, (B) plasma spiked with MMF (145 ng/mL), MPA (40 ng/mL) and internal standard, and (C) a plasma sample obtained from a patient (MMF iv).

MMF, MPA, and MPAG are 0.400, 0.100, and 4.00 $\mu\text{g/mL}$, respectively. Depending on the assay, the plasma volume needed ranges from 0.2 to 0.5 mL of plasma. Furthermore, most of the published methods are based on a solid-phase extraction (C-18 cartridges) of MPA and MPAG, which is time-consuming and

Table 1. Intraday Precision Data for MMF and MPA^a

nominal concn (ng/mL)	mean concn (ng/mL)	RSD (%)	mean recovery (%)
MPA			
3.12	3.05	1.81	97.52
6.25	6.11	1.16	97.79
12.50	11.95	2.16	95.63
25.00	24.60	1.07	98.41
50.00	48.04	3.02	96.07
100.00	95.94	1.22	95.94
150.00	147.81	1.61	98.54
200.00	197.00	2.76	98.50
1000.00	1001.45	1.14	100.15
MMF			
15.62	16.16	3.29	103.47
31.25	30.90	2.17	98.89
62.50	59.74	0.76	95.58
125.00	127.20	1.33	101.76
250.00	247.58	1.37	99.03
500.00	497.15	1.02	99.43
750.00	743.85	0.82	99.18
1000.00	1006.47	1.14	100.65

^a The intraday precision ($n = 4$) of MMF and MPA calibration curves dissolved in plasma.

expensive. Another very simple method is the immunoassay for the detection of MPA, which detects MPA photometrically (340 nm). Compared with this method, the described HPLC assay is over 4 orders of magnitude more sensitive.²³ Due to the ease of performance and the speed, the method described here appears suitable for large-scale analyses and monitoring of patients.

The pharmacologically inactive metabolite MPAG is hydrolyzed in vivo to MPA. To enable fluorescence detection, this metabolite was decomposed to MPA enzymatically. During the catalyzed hydrolysis reaction in a water bath at 37 °C, other plasma enzymes are also active. Esterases present in human plasma may catalyze the deesterification of MMF. Therefore, an inhibition of esterases was investigated. However, detailed analyses showed that time-intensive sample preparation procedures are necessary to prevent the enzymatic ester hydrolysis (data not shown). These are unsuitable for routine analysis. A very simple and efficient procedure was used to determine the plasma levels of MPAG, applying the data of both HPLC chromatographic analyses (before and after the deglycuronidation reaction). Comparing the MMF level (pre- and postincubation), the MPA values obtained were corrected for the deesterification of MMF (see formula described in the Experimental Section).

Reproducibility and Recovery. The reproducibility of the assay was assessed by comparison of the standard deviation of intraday and interday values (RSD (%)). The analysis of calibration curves for MPA and MMF ($n = 4$, within-day) resulted in RSD values of 3.02 (MPA in plasma) and 3.29% (MMF in plasma) (Table 1). The RSD data for the corresponding interday analysis are presented in Table 2. The recoveries of MPA and MMF from plasma (intraday data) were determined by the analysis of plasma spiked with MPA and MMF. The recovery ranged from 94.91 to 102.88% (Table 1). These data confirmed the accuracy of our assay. Very

- (19) Jones, C. E.; Taylor, P. J.; Johnson, A. G. *J. Chromatogr., B* **1998**, 708, 229–234.
- (20) Gummert, J. F.; Christians, U.; Barten, M.; Silva, H.; Morris, R. E. *J. Chromatogr., B* **1999**, 721, 321–326.
- (21) Svensson, J. O.; Brattstrom, C.; Sawe, J. *Ther. Drug Monit.* **1999**, 21, 322–324.
- (22) Shipkova, M.; Schutz, E.; Armstrong, V. W.; Niedmann, P. D.; Oellerich, M.; Wieland, E. *Clin. Chem.* **2000**, 46, 365–372.

- (23) Haley, C. J.; Staples, M. A.; Vorpahl, J.; Wieder-Bunger, T. A.; Jaklitsch, A. P. Poster presented at 49th Annual AACC Meeting, Atlanta, GA, July 20–24, 1997.

Table 2. Interday Precision Data for MMF and MPA^a

nominal concn (ng/mL)	mean concn (ng/mL)	RSD (%)	mean recovery (%)
MPA			
3.12	3.09	5.18	98.91
6.25	6.03	4.08	96.46
12.50	13.83	6.40	110.64
25.00	25.34	5.94	101.35
50.00	51.81	2.19	103.63
100.00	94.97	2.82	94.97
150.00	149.08	4.31	99.38
200.00	202.62	3.38	101.31
1000.00	997.37	1.49	99.74
MMF			
15.62	14.77	4.36	94.59
31.25	30.16	5.57	96.50
62.50	61.28	4.72	98.05
125.00	122.06	6.47	97.65
250.00	247.67	5.39	99.07
500.00	493.61	2.16	98.72
750.00	751.69	1.06	100.23
1000.00	1003.06	2.66	100.31

^a The interday precision ($n = 5$) of MMF and MPA calibration curves dissolved in plasma.

similar results were obtained for MPA and MMF dissolved in eluent system (data not shown).

Linearity. The calibration curve is linear over 3 orders of magnitude with correlation coefficients of 0.999 (MPA) and 0.998 (MMF).

Limit of Quantification. The limits of quantification for both MPA and MMF are 3.3 and 4.4 ng/mL respectively, extracting, for example, a 0.1-mL plasma sample. At this concentration, the

S/N were 5:1 for MPA and MMF. Regarding the sensitivity, no differences between the spiked plasma samples and the HPLC eluent samples were observed.

Specificity. Our assay shows a high specificity caused by the kind of detection. Additionally, we evaluated the specificity of the described method by investigation of the peak response produced by drugs that might be potentially coadministered with MMF. The following drugs did not interfere due to their retention time or did not show fluorescence under the assay conditions described: cyclosporine A, methotrexate, prednisone, methylprednisolone, cimetidine, and ganciclovir.

CONCLUSION

The solvatochromic reaction of an analyte provides an efficient tool for lowering the optical detection limit of an analyte. Using this effect, here we show that a high level of sensitivity can be obtained in the detection of MPA, MMF, and MPAG by postcolumn derivatization. Although such a sensitivity in detection of the drug was unknown up to now, the handling of the assay is simple and robust. Therefore, the method is suitable for both clinical studies²⁴ and the daily clinical routine. Due to its extreme sensitivity, the assay may also provide a useful tool for drug analysis in very small tissue extracts, for example, analysis in lymphocyte subpopulations. For the described setup of the postcolumn derivatization, an electronically regulated valve controlling the flow of the reactant solution under a pulsation-free gas pressure is used. This arrangement enables a variety of different derivatization reactions under various chemical conditions (e.g., temperature, concentration, or viscosity of the reactant solution).

Received for review June 14, 2000. Accepted October 17, 2000.

AC0006730

(24) Bornhäuser, M.; Schuler, U.; Pörksen, G.; Naumann, R.; Geissler, G.; Thiede, C.; Schwerdtfeger, R.; Ehninger, G.; Thiede, H.-M. *Transplantation* **1999**, *67*, 1–6.