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Determination of mycophenolic acid and its phenol glucuronide metabolite in human plasma and urine by high-performance liquid chromatography

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Abstract

Simultaneous determination of mycophenolic acid (MPA) and mycophenolate phenol glucuronide (MPAG) in plasma and urine was accomplished by isocratic HPLC with UV detection. Plasma was simply deproteinated with acetonitrile and concentrated, whereas urine was diluted prior to analysis. Linearity was observed from 0.2 to 50 μg/ml for both MPA and MPAG in plasma and from 1 to 50 μg/ml of MPA and 5 to 2000 μg/ml MPAG in urine with extraction recovery from plasma greater than 70%. Detection limits using 0.25 ml plasma were 0.080 and 0.20 μg/ml for MPA and MPAG, respectively. The method is more rapid and simple than previous assays for MPA and MPAG in biological fluids from patients. © 2001 Published by Elsevier Science B.V.

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1. Introduction

Mycophenolic acid (MPA), originally isolated from *Penicillium* culture in 1898, has antibiotic, antifungal, antineoplastic, antipsoriatic, and immunosuppressive activities [1–12]. With the discovery that MPA inhibits de novo purine biosynthesis, it is now employed as a lymphocyte-specific immunosuppressive agent approved for use in humans. MPA noncompetitively and reversibly binds to inosine monophosphate dehydrogenase (IMPDH), a rate-limiting enzyme in de novo guanine biosynthesis [13,14]. Lymphocytes primarily depend on this pathway, thus the reduction of guanosine nucleotides synthesis results in suppression of T and B lymphocyte proliferation.

MPA is marketed in the US as a morpholinoethyl ester prodrug, mycophenolate mofetil (MMF), to improve the bioavailability of MPA [15]. Oral and intravenous formulations are available, but capsule is the most commonly used dosage form [16]. MMF is rapidly absorbed and hydrolyzed to MPA, which is further metabolized to form a pharmacologically inactive phenolic glucuronide, MPAG [17–19]. Cur-

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rently, MMF is approved for prophylaxis of graft rejection in patients receiving allogenic renal transplants [20]. It is used in combination with other immunosuppressive agents in other transplant types and immunological related disorders [21,22]. Most pharmacokinetic analyses of MMF have been conducted in renal transplant patients, whereas experience with MMF in other transplant populations is still limited. Data also suggested that enterohepatic recycling of MPA in humans, a primary determinant of MPA disposition, might be altered in disease and other conditions leading to large interindividual pharmacokinetic (PK) variability [18]. Therapeutic drug monitoring of MPA as well as PK studies in populations with high variability, such as transplant or pediatric patients, are needed. To investigate the pharmacokinetics of MPA in patient populations, a rapid and simple analytical method that can accurately determine both MPA and MPAG in biological samples was developed and is reported here. Analysis of MPAG is of interest, as this primary metabolite achieves plasma levels many fold higher than MPA and is involved in enterohepatic recycling of MPA which results in secondary peaks in the concentration versus time profile and prolongs the apparent half-life of MPA in vivo.

Several analytical methods for MPA have been established to support pharmacokinetic studies in human or animal models. The earliest method for MPA analysis was reported in 1972 where MPAG was indirectly quantified [23]. Due to the fact that MPAG cannot be easily analyzed using gas chromatography, this early method required hydrolysis of the sample with β-glucuronidase to quantify a total amount of MPA [23]. A number of HPLC methods to analyze MPA and MPAG in biological samples were developed more recently for pharmacokinetic studies [24–29]. However, by reason of the large difference in polarity of MPA and MPAG, some earlier HPLC methods require a separate HPLC analysis, two analytical columns and mobile phase systems for analysis of each compound in plasma [25]. Although an automated operation using robotic system was suggested for the sequential dual analytical columns, the system is unnecessarily complex [25]. These previously described methods were laborious and less applicable for high throughput or clinical analysis. A direct and simultaneous determination of MPA and MPAG was developed here to provide a rapid and accurate method for routine analysis of biological samples from clinical studies. An immunoassay method using EMIT TM technique was also recently reported [30]. However, the method revealed an overestimation of MPA concentration in plasma from liver and kidney transplant patients [30] by EMIT assay when compared to an HPLC method. The investigator attributed this overestimation to a cross reactivity of an alternate metabolite later identified as MPA acyl glucuronide [31]. A more recent analytical method from the same laboratory extended the analysis by using a gradient HPLC method to include MPA acyl glucuronide, a minor metabolite, and described methods to stabilize the labile acyl glucuronide by adjusting the pH of plasma upon collection [32].

Other liquid chromatographic techniques allowing for determination of MPA and MPAG simultaneously were published recently [27–29]. These methods employed sample preparation procedures [27–29] and mobile phase gradient elution [27,29] which are generally more complex than the procedures described here. The present isocratic method proved to be fast and reliable for simultaneously analysis of MPA and MPAG using a single analytical column and mobile phase system. Based on the simple sample preparation and isocratic chromatography, improvement was obtained not only by increasing sample throughput, but also decreasing the cost of the assay and instrumentation required.

2. Materials and methods

2.1. Chemicals and reagents

MPA was purchased from Sigma (St. Louis, MO, USA). MPAG was synthesized according to the method reported by Ando et al. [26]. Suprofen, a lipophilic acid with comparable UV absorption to MPA, was obtained from the Pharmaceutical Research Institute (Springhouse, PA, USA, USA) and was employed as an internal standard (I.S.) for the assay. Acetonitrile (HPLC grade) was from J.T. Baker (Phillipsburg, NJ, USA). Methanol was from Mallinckrodt Baker (Paris, KY, USA). Trifluoroacetic acid was from Sigma. Pooled blank human

plasma was obtained from the blood bank of the University of North Carolina Hospital. Blank human urine was from a healthy volunteer after an overnight fast. Other drugs tested for potential interference with the assay were obtained from various sources, chemical suppliers or from dosage forms from the university hospital.

MPA (1.0 mg/ml in methanol) and MPAG (5.0 mg/ml in 50% methanol) were prepared separately as stock solutions and stored at −20°C. Serial dilution yielding 0.10, 0.010 and 0.001 mg/ml of MPA and MPAG were prepared in 50% methanol–water. Suprofen, used as the I.S., was prepared (1.0 mg/ml) as a solution in methanol and diluted 10 fold for addition to biological samples.

2.2. Synthesis of mycophenolic acid-β-D-glucuronide

MPAG was synthesized according to the method reported by Ando et al. [26] with slight modification. MMF, extracted from the capsules (CellCept®, Roche) and obtained as a white crystal, was used as the substrate in the condensation with methyl (tri-O-acetyl-α-D-glucopyranosyl bromide)-uronate. MMF (1 g) was dissolved in 100 ml pyridine before 0.6 g silver carbonate was added. Stirring in the dark for 20 min, 1 g methyl (tri-O-acetyl-α-D-glucopyranosyl bromide)-uronate was added. The reaction mixture was continued for 10–12 h with stirring, then it was diluted with 200 ml of toluene and filtered through Celite. The organic layer was then washed sequentially with 2×200 ml of 1 *M* HCl, 2×200 ml of 0.3 *M* KOH, and 2×200 ml of water. With the subsequent addition of magnesium sulfate to the mixture for drying, the product was obtained after evaporation of the toluene layer.

The crude product was subjected directly without purification to further saponification and deacetylation. This was carried out in 20 ml acetone by adding 20 ml 1 *M* NaOH and maintaining the solution at room temperature. After stirring for 15 min, Amberlite IR-120 (H+ form) was added to remove sodium ion. After filtering, the resin was washed with 2×30 ml toluene. The residue after evaporation of the organic solvent was redissolved with the minimum amount of ethanol with continuous heating. The clear yellowish ethanolic solution was added to a small volume of petroleum ether. The solution was kept at −20°C overnight, yielding MPAG.

Under the assay conditions, synthesized MPAG exhibited one major peak on HPLC with UV detection at 250 nm. The eluted peak had a purity of 96–98%, with the remaining 2–4% as polar impurities that eluted earlier. Characterization of MPAG was done by mass spectrometry using electrospray ionization in the positive ion mode, elemental analysis, susceptibility to β-glucuronidase and resistance to hydrolysis with 0.1 *M* sodium hydroxide using methods previously described [33].

2.3. Instrumentation

The HPLC system consisted of a Pharmacia LKB 2150 pump (Bromma, Sweden), Bio-Rad model AS-100 automatic injector, Hewlett-Packard Series 1050 UV detector, and Axxiom ODS column (150×4.6 mm I.D., 5 μm) (Springfield, VA, USA) connected to a RP-18 guard column (15×3.2 mm I.D., 7 μm) (Brownlee, San Jose, CA, USA). A computer interface was linked to a Hewlett-Packard CHEMSTATION A.05.01. CHEMSTATION was used for recording and storing the data throughout the analysis. Methanol–trifluoroacetic acid (TFA, 0.1% w/v) (48:52) was used as the mobile phase at a flow-rate 1.5 ml/min. The compounds were quantified by UV detection at 250 nm.

2.4. Standard curve for analysis of MPA and MPAG in plasma

MPA and MPAG concentrations used in calibration curves were based upon mass/volume, e.g. μg/ml, using standards, then MPAG concentrations were later expressed as MPA equivalents when applied to concentration vs. time profiles. Aliquots of 0.25 ml blank human plasma were spiked with 5–25 μl stock solutions of MPA or MPAG yielding final concentrations of 0.2, 0.5, 1.0, 4.0, 10, 20, 50 μg/ml after mixing. The solutions were allowed to equilibrate for 10 min at room temperature, then 30 μl of suprofen solution (0.10 mg/ml) was added as I.S., followed by addition of 0.75 ml of acetonitrile to precipitate proteins. Samples were then vortexed for 1–2 min and centrifuged at 3000 *g* for 10 min at 4°C. The supernatant was decanted into a clean test tube and

evaporated to dryness under nitrogen stream at 40°C. After reconstitution with 0.2 ml of 25% methanol in 10 m*M* TFA, the samples were transferred to polypropylene tubes and centrifuged at 4000 *g* for 10 min to remove solids remaining. Aliquots of 15–60 μl of the clear supernatant were then injected onto the analytical HPLC.

2.5. Standard curve for analysis of MPA and MPAG in urine

Aliquots (25-μl) of blank human urine were spiked with 12–50 μl stock solutions to yield final concentrations of 5, 25, 100, 200, 500 μg/ml for MPAG, and 1, 5, 10, 20, 50 μg/ml for MPA. A 75-μl volume of suprofen solution (0.1 mg/ml) was added, then the solutions were diluted to 0.5 ml with 25% methanol in 10 m*M* TFA. Aliquots (15–85 μl) of the diluted solutions were injected into the HPLC system.

2.6. Application of the method for the analysis of clinical samples

Six healthy subjects enrolled in a clinical study, approved by the University IRB, were each given a single oral 1-g dose of MMF. Blood samples were collected over 48 h from each subject into heparinized (50 U/ml) tubes. Blood samples were immediately centrifuged in a clinical centrifuge, 4°C for 10 min to separate plasma. Plasma was transferred to screw top vials and stored at −20°C until analysis. Urine samples were collected incrementally over the same 48-h study period. After determining the total volume, 5–10 ml aliquots of urine were obtained and stored at −80°C until analysis. For analysis, the plasma or urine samples were thawed and treated as described above. MPA concentrations were reported directly in μg/ml, whereas MPAG concentration was measured against authentic synthesized MPAG. All MPAG concentrations were then reported in terms of μg/ml MPA-equivalents, obtained by multiplying each MPAG concentration by 0.646 (the ratio of molecular mass of MPA to MPAG).

Several humans subjects undergoing liver transplantation and a normal volunteer were also studied, where plasma samples were divided upon collection, with one aliquot adjusted to pH<4 by the addition of phosphoric acid, while the other served as a control. These plasma samples were analyzed as described above, with the only change being the use of acidified plasma for constructing the standard curve for those samples acidified upon collection. The results of the acid treated vs. control plasma samples was compared for MPAG and MPA concentrations, since it was reported that putative MPA acyl glucuronide was stabilized by acidification [32], similar to that observed for other acyl glucuronides [33,34]. An ANOVA was applied to determine if there was a difference in results between the acid treated vs. control plasma samples with respect to MPA or MPAG concentrations.

2.7. Validation of plasma and urine sample analysis

Intra-day, inter-day assay precision, as well as recovery for plasma sample analysis of MPA and MPAG were studied using pooled blank plasma. MPA and MPAG were added to blank plasma to yield concentrations of 1, 10, and 50 μg/ml for MPA, and concentrations of 1 and 10 μg/ml for MPAG. Spiked plasma, 0.5 ml, was aliquoted into screw cap vials and stored at −80°C until analysis. Sample preparations and analysis procedures followed that of the standard curve described above. Inter-day validation was performed by repeated analysis of these samples over a 6-month period. Intra-day validation was conducted by preparing 5–6 separate samples at each concentration and analyzing them in a single day.

For assessment of precision and accuracy for urine sample analysis, stock solutions of MPA and MPAG were added to blank human urine to achieve final concentrations of 2.0 μg/ml for MPA, and 25, 200 and 1000 μg/ml for MPAG. These samples were stored at −80°C until analysis. A 25-μl volume of these solutions was added with suprofen and diluted to 0.5 ml as previously described above. Five to six separate samples at each concentration were analyzed in a single day for intra-day assay precision, and inter-day precision was conducted over a 3-month period.

Extraction recovery of compounds from plasma was evaluated by comparing peak area ratios (MPA or MPAG to suprofen) obtained from the extracted

Table 1
Drugs potentially co-administered with MMF that were examined in the analytical method for possible interference with the HPLC assay

Immunosuppressives	GI drugs
Methylprednisolone	Ranitidine
Cyclosporin	Cisapride
Tacrolimus	Omeprazole
Antibiotics	Antihypertensives
Ciprofloxacin[a]	Metoprolol
Meronidazole[a]	Atenolol
Sulfamethoxazole–trimethoprim	Felodipine
	Amlodipine
NSAIDs	Clonidine
Acetaminophen	Diltiazem
Salicylate[b]	Furosemide
Ibuprofen	
	Antivirals
Antifungals	Acyclovir
Fluconazole	Ganciclovir

[a] Drugs given concomitantly in healthy subject study.
[b] Drugs showed interferences with MPAG.

plasma sample containing either MPA or MPAG with area ratios obtained from unextracted standard compounds prepared in 25% methanol–TFA that were injected directly onto HPLC. Suprofen was used as an external standard to determine the recoveries.

The specificity of the assay was evaluated, as multidrug use is quite common in patients undergoing organ transplantation. The potential interferences from co-administered drugs, as listed in Table 1, were examined. Drugs, listed in Table 1, were prepared in 50% methanol solutions to yield final concentration of each drug at 5 and 50 μg/ml, and analyzed by HPLC. Plasma samples from transplant patients receiving immunosuppressive therapy with MMF were also analyzed by the procedures previously described. Furthermore, the described method was also applied for the analysis of plasma samples from an ongoing pharmacokinetic study of the effect of antibiotics concomitantly administered with MMF in healthy subjects.

3. Results

Synthesized MPAG was characterized as a β-1 phenolic glucuronide conjugate. This was determined using the following criteria: (1) stability against hydrolysis in the presence of strong base, (2) susceptibility to cleavage by β-glucuronidase, and (3) β-glucuronidase hydrolysis was inhibited by D-saccharic acid 1,4-lactone. In addition, elemental analysis of MPAG ($C_{23}H_{28}O_{12}$) was found to contain C=55.7%, H=5.72% (theoretical C=55.6% and H=5.68%). Electrospray LC–MS confirmatory identification of MPAG revealed m/z 520 corresponding to $[M_{MPAG}+Na^+]$ and a base peak at m/z 321 corresponding to $[M_{MPA}+H^+]$ of MPA after loss of glucuronic acid.

Fig. 1 shows representative chromatograms of blank plasma, a plasma sample containing 10 μg/ml MPA and MPAG, and a plasma sample at 1.0 h after a healthy subject received a single 1 g oral dose of MMF. The retention time of MPAG, suprofen, and MPA were approximately 3.7, 9.4, and 14.6 min, respectively, with a total analysis time of 22 min to avoid late eluting variable endogenous peaks for each chromatographic run. Fig. 2 are chromatograms of representative blank urine, urine samples containing 10 μg/ml of MPA and 100 μg/ml of MPAG, and a urine sample at 0–4 h after a healthy subject received a single 1 g oral dose of MMF. Plasma and urine samples from healthy subjects who received norfloxacin and metronidazole concomitantly with MMF, revealed no additional endogenous peaks or interferences in the chromatograms. Most of the commonly co-administered compounds, listed in Table 1, did not show any interfering peaks under the assay condition described except salicylate, which eluted at a similar retention time as MPAG.

Calibration curves were linear over the range of 0.2–50 μg/ml MPA and MPAG for plasma samples, and 1–50 μg/ml MPA, and 5–500 μg/ml MPAG for urine samples based on the peak area ratio of MPA or MPAG to suprofen. Correlation coefficients greater than 0.995 for both plasma and urine were routinely obtained when weighted using the reciprocal of the concentrations. Intra- and inter-day reproducibilities for plasma and urine samples assay are shown in Tables 2 and 3, respectively. In plasma, the relative standard deviation (RSD) for MPA and MPAG ranged from 2.4 to 9.3% and 5.9 to 11% for intra- and inter-day variabilities, respectively (Table 2). Reproducibilities for urine sample analysis showed intra-day variabilities from 1.3 to 6.0% for MPA and MPAG (Table 3). The accuracy of the assay of MPA

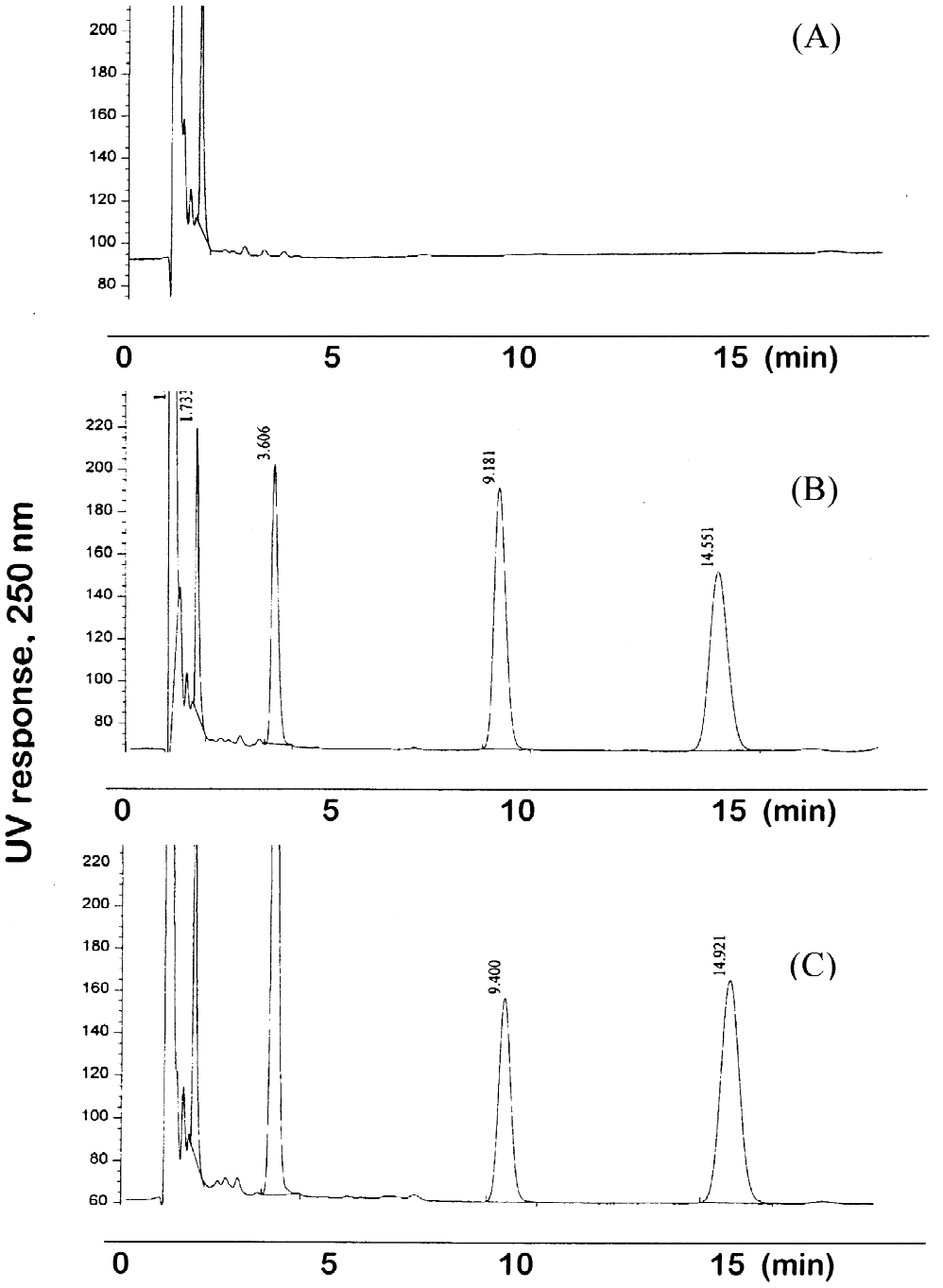

Fig. 1. HPLC chromatograms of MPA and MPAG in human plasma. (A) Blank plasma; (B) blank plasma spiked with MPA, MPAG (10 μg/ml) and internal standard, suprofen; (C) plasma sample at 1.0 h from a subject administered a single 1 g oral dose of MMF. Retention times: MPAG~3.6 min, Suprofen (I.S.)~9.2 min, MPA~14.6 min. Units of ordinate are arbitrary.

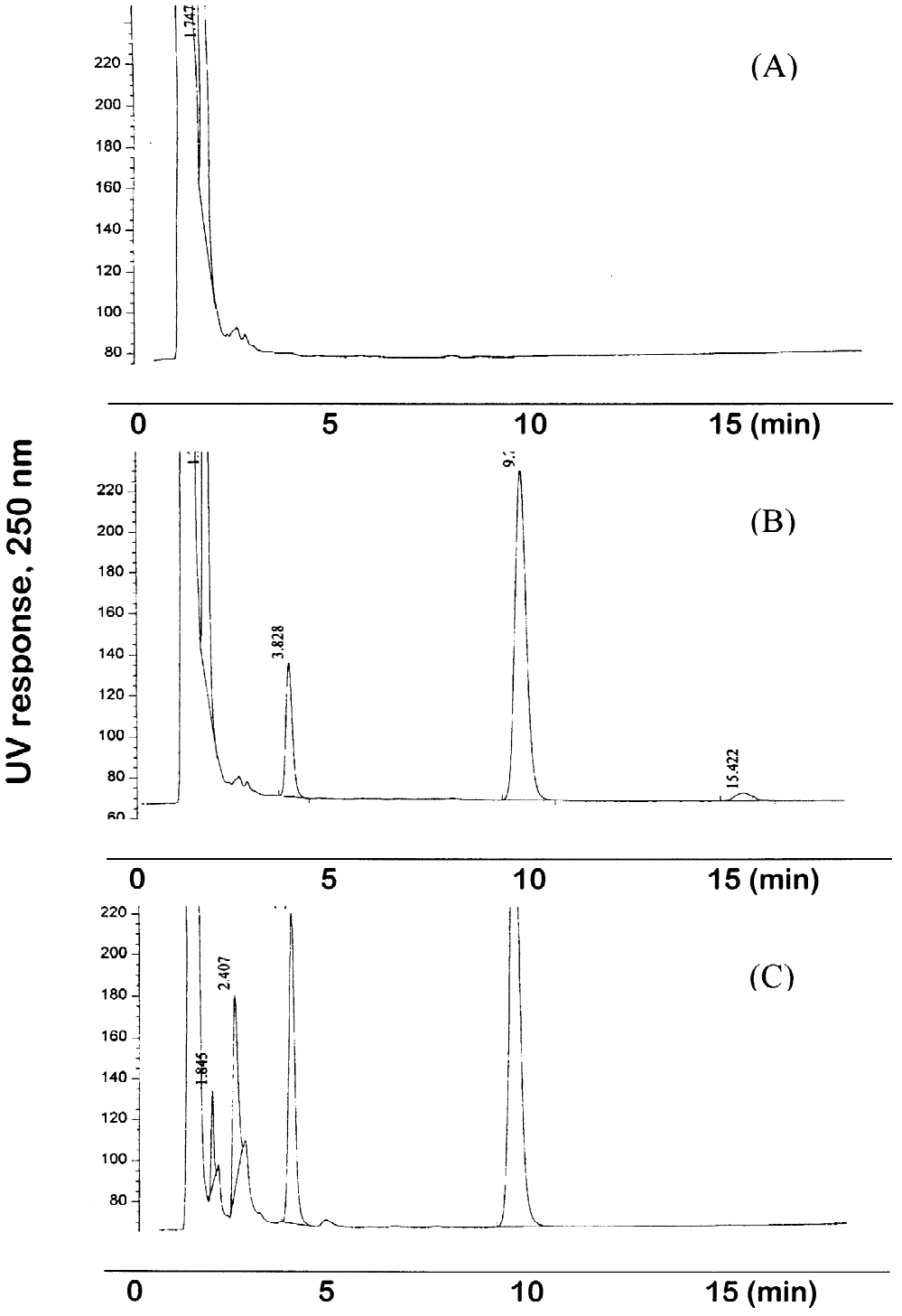

Fig. 2. HPLC chromatograms of MPA and MPAG in human urine. (A) Blank urine; (B) blank urine spiked with MPA (10 μg/ml) and MPAG (100 μg/ml); (C) urine sample at 0–4 h from a subject administered a single 1 g oral dose of MMF. Retention times: MPAG~3.8 min, Suprofen (I.S.)~9.7 min, MPA~15.4 min. Units of ordinate are arbitrary.

Table 2
Intra-day and inter-day variabilities for the analysis of MPA and MPAG in plasma

Plasma conc. (μg/ml)	Concentrations found, μg/ml, mean±SD (RSD, %)			
	MPA		MPAG[c]	
	Intra-day[a]	Inter-day[b]	Intra-day	Inter-day
1.0	0.95±0.9 (9.3)	1.04±0.1 (8.0)	1.1±0.0 (3.7)	0.97±0.1 (11)
10.0	8.72±0.2 (2.4)	9.51±0.9 (9.6)	10.4±0.3 (2.6)	10.1±0.6 (5.9)
50.0	49.3±1.9 (3.9)	55±4.8 (8.8)	N.D.	N.D.

[a] $n=5$–6.
[b] $n=8$.
[c] MPAG expressed as MPA equivalents.

and MPAG in plasma and urine was generally between 90 and 110% (Tables 2 and 3).

As shown in Table 4, recovery from plasma was 82–88% and 76–90% for MPA and MPAG, respectively, over the range of 1–50 μg/ml. The limit of detection (LOD) in plasma with was 0.08 μg/ml for MPA and 0.2 μg/ml MPAG at 250 nm, based upon the lowest tested concentration yielding <15% RSD of inter-day precision. In urine samples, LOD was found to be 1 μg/ml for MPA and 5 μg/ml for MPAG.

The assay method has been applied to the analysis of MPA and MPAG in greater than 1000 plasma and urine samples from healthy subjects and animals administered MMF. The concentration–time profile for MPA and MPAG in plasma obtained from six healthy subjects participating in an ongoing pharmacokinetic study are shown in Fig. 3. The results indicate that maximum MPA concentration of 15.5 (±9.2) μg/ml was obtained at 0.5 h after oral administration of 1 g MMF and declined rapidly afterward. MPA concentrations were in the range of 0.2–6.0 μg/ml between 1.5 and 48 h after dosing. Maximum MPAG concentration, obtained at 1.5 h was found to be 25.5 (±10.3) μg/ml after dosing and MPAG levels were higher than MPA at every time point. Urinary MPAG excretion over 48 h was found to be 83(±8.8)% of the total administered dose, with the maximum excretion obtained during the 0–4 h interval.

Adjusting the plasma samples to pH<4 provided no discernable difference in measured MPA or MPAG in plasma for the normal volunteer and the

Table 4
Extraction recovery of MPA and MPAG from plasma

Plasma conc. (μg/ml)	Recovery, %, mean±SD[a] (RSD, %)	
	MPA	MPAG
1.0	88±4.2 (4.7)	90±7.7 (8.6)
10.0	86±6.4 (7.4)	77±5.3 (6.9)
50.0	82±1.1 (1.4)	80±2.5 (3.1)

[a] $n=6$.

Table 3
Intra-day and inter-day variabilities for the analysis of MPA and MPAG in urine

Urine conc. (μg/ml)	Concentrations found, μg/ml, mean±SD (RSD, %) MPA		MPAG[c]	
	Intra-day[a]	Inter-day[b]	Intra-day	Inter-day
2.00	2.16±0.1 (5.3)	2.12±0.3 (13)	N.D.[d]	N.D.
25.0	N.D.	N.D.	26.1±0.7 (2.7)	27.9±1.2 (4.3)
200	N.D.	N.D.	208±12 (6.0)	204±2.9 (1.4)
1000	N.D.	N.D.	1073±14 (1.3)	1011±41 (4.1)

[a] $n=5$–6.
[b] $n=5$.
[c] MPAG expressed as MPA equivalents.
[d] N.D., not done.

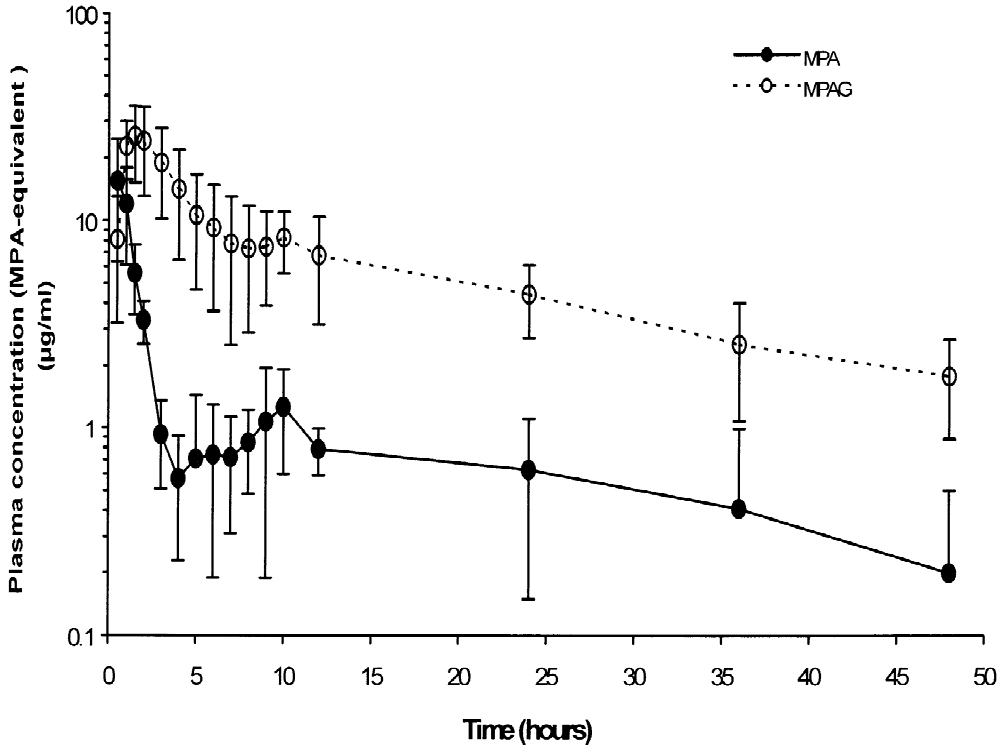

Fig. 3. Average plasma concentration–time profile of MPA and MPAG from healthy subjects ($n=6$) administered a single 1-g oral dose of MMF. Solid circle, MPA; open circle, MPAG. Bars indicate SD of mean.

three subjects who recently received a liver transplant.

4. Discussion

This assay method was developed to quantify both MPA and MPAG in biological samples using a single chromatographic run for application to pharmacokinetic studies or therapeutic drug monitoring. To date, three recent HPLC methods have been reported for simultaneously determination of MPA and MPAG in plasma samples. The retention of MPA described here is similar to the method employed by Huang et al. [27]. However, MPAG shows better separation from endogenous peaks using the presently described analytical conditions when capacity factors (k') are considered. The components of the mobile phase employed herein are simpler than the conditions recently reported by Shipkova et al. [29] with the advantage of a volatile trifluoroacetic acid mobile phase for coupling to MS, if desired, for structural confirmation. The protein precipitation procedure described here also allows for faster sample processing than previously described methods. Using an isocratic mobile phase the total analysis time was 22 min to avoid late endogenous peaks eluted after MPA (t_R 15 min).

The test for interfering peaks from drugs potentially administered with MMF resulted in co-elution of MPAG and salicylate. Evaluation of medication history to reveal co-administered with salicylate is suggested to avoid possible assay interference when the research protocol requires that MPAG be monitored.

The absorption spectrum of MPA under the assay conditions revealed three maxima at 215, 250 and 306 nm, while MPAG showed absorption maxima at 215, 250 and 294 nm. Quantification was performed at 250 nm since both compounds had absorption

maxima at this wavelength. Analysis at 215 nm was not feasible due to significant interference from endogenous material. The higher wavelength corresponded to the third maxima of both compounds, however, the absorptivity decreased significantly at this wavelength, i.e. A_{250}:$A_{294} \approx 3$ for MPAG and A_{250}:$A_{306} \approx 2$ for MPA, which may lead to loss of sensitivity. Considering the ~3-fold higher absorptivity of MPA over MPAG at 250 nm, detection performed at this wavelength is reasonable since MPAG is present in plasma samples at higher levels than of MPA at any time point during the 12–48 h interval post-dose.

Sensitivity attained by this method (0.08 μg/ml for MPA and 0.2 μg/ml for MPAG) was improved compared to the previously described method [27], which also employed a larger plasma volume. The detection limit of the present method, however, is not as low as that reported by Shipkova et al. [29], where 0.03 μg/ml for MPA and 0.1 μg/ml for MPAG, were measurable using 0.2 ml plasma. In addition to clinical sample analysis, where 0.25 ml plasma was used, the method described here was applied to plasma samples from animal studies where only 0.1 ml plasma volume was available. The assay method described here was also adequate for analysis of bile samples from animals when UV detection was performed at 295 nm to increase specificity. Bile samples are predominantly MPAG and have concentrations much higher than seen in plasma.

Storage and analysis of the control plasma and urine samples over several months provided no evidence of apparent change of MPA or MPAG concentrations when −20°C was employed for storage. This is in agreement with MPA stability in biological fluids as reported earlier by Tsina et al. [25] who indicated that it was stable in whole blood and plasma for at least 8 h at 1–4°C and at least 11 months at −20°C.

The addition of acid to plasma to stabilize putative MPA acyl glucuronide provided no change in measurable MPA concentrations. This may be due to possible low levels of MPA acyl glucuronide relative to MPA, in contrast to MPAG that accumulates to concentrations many times that of MPA. Shipkova et al. [32] reported a median level of the putative labile metabolite of 0.75 μg/ml, however, relevant details such as the level of this metabolite relative to parent, MPA, the time of sampling, type of patient and doses employed were not provided. If present, the labile metabolite would probably reach higher concentrations in subjects with renal failure, though its accumulation relative to MPA would likely be less than what is observed for MPAG [16,35] because acyl glucuronides are subject to hydrolysis via esterases and futile cycling in vivo [36,37]. Whether metabolite levels would be dependent upon renal function was not discussed by Shipkova et al. [32], though their sample chromatogram from plasma of a renal transplant patient showed an MPA acyl glucuronide:MPA ratio of 1.5 together with an MPAG:MPA ratio of 121, with the later ratio suggestive of a patient with severe renal dysfunction. This distinction is important, as reports of MPA disposition in patients with poor renal function may have significant error due to overestimation of MPA plasma concentration because of possible hydrolysis of labile MPA acyl glucuronide. In contrast, the earlier reports of MPA disposition in subjects with moderate or good renal function may have very little potential error, as the labile acyl glucuronide metabolite would not be expected to accumulate in plasma. We found no difference in chromatography profiles or concentrations of MPA and MPAG when plasma samples were pretreated with acid to stabilize putative MPA acyl glucuronide. Therefore, treatment to prevent possible degradation of MPA acyl glucuronide is not necessary for plasma collected from subjects with normal renal function, but such pH adjustment is a reasonable approach as it removes the uncertainty of renal function on the assay for MPA.

In conclusion, the HPLC method presented here is simple, accurate, and reliable for the simultaneous determination of MPA and MPAG in human plasma and urine using 0.25 ml of sample. With minor modification it has also been applied to animal studies with smaller sample volumes of 0.1 ml. The analytical method has been successfully applied to the analysis of biological fluids obtained from patients receiving MMF for immunosuppression following organ transplantation. There was no evidence of altered plasma concentrations of MPA or MPAG in patients with adequate renal function when efforts were taken to prevent degradation of a putative minor metabolite, MPA acyl glucuronide. Most common concomitant drugs did not interfere with the

assay, however, salicylate may prevent accurate measurement of MPAG with the method. The analytical method is rapid and simple, thus it should be easily adaptable in many laboratories using commonly available HPLC equipment.

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