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Journal of Chromatography B, 799 (2004) 157–163
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Quantification of free mycophenolic acid and its glucuronide metabolite in human plasma by liquid-chromatography using mass spectrometric and ultraviolet absorbance detection

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Received 23 June 2003; received in revised form 10 October 2003; accepted 20 October 2003

Abstract

The immunosuppressant drug mycophenolic acid (MPA) and its major metabolite, mycophenolic acid glucuronide (MPAG), are highly bound to albumin. An HPLC-tandem-MS (HPLC/MS/MS) and an HPLC-UV assay were developed to measure free (unbound) concentrations of MPA and MPAG, respectively. Ultrafiltrate was prepared from plasma (500 μl) by ultrafiltration at 3000 × *g* for 20 min (20 °C). Both MPA and MPAG were isolated from ultrafiltrate (100 μl) by acidification and C_{18} solid-phase extraction. Free MPA was measured by electrospray tandem mass spectrometry using selected reactant monitoring (MPA: *m/z* 338.2 → 206.9) in positive ionisation mode. Chromatography was performed on a PFPP column (50 mm × 2 mm, 5 μm). Total analysis time was 7 min. The assay was linear over the range 1–200 μg/l with a limit of quantification of 1 μg/l. The inter-day accuracy and imprecision of quality controls (7.5, 40, 150 μg/l) were 94–99% and <7%, respectively. Free MPAG was chromatographed on a C_{18} Nova-Pak column (150 mm × 3.9 mm, 5 μm) using a binary gradient over 20 min. The eluent was monitored at 254 nm. The assay was linear over the range 1–50 mg/l with the limit of quantification at 2.5 mg/l. The inter-day accuracy and imprecision of quality controls (5, 20, 45 mg/l) was 101–107% and <8% ($n = 4$), respectively. For both methods no interfering substances were found in ultrafiltrate from patients not receiving MPA. The methods described have a suitable dynamic linear range to facilitate the investigation of free MPA and MPAG pharmacokinetics in transplant patients. Further, this is the first reported HPLC-UV method to determine free MPAG concentrations.

Keywords: Mycophenolic acid; Glucuronide; Free drug

1. Introduction

Mycophenolic acid (MPA) is the active moiety of the registered prodrug mycophenolate mofetil (MMF). MMF is used in renal transplant patients for the prophylaxis of acute rejection. When taken orally, MMF is rapidly hydrolysed to MPA. MPA is metabolized by glucuronidation (>80%), forming the major metabolite mycophenolic acid glucuronide (MPAG) and a minor acyl glucuronide metabolite. MPAG may undergo entero-hepatic cycling or is excreted in the urine [1]. Plasma MPAG concentrations are at least an order of magnitude greater than those of MPA in renal transplant recipients [2].

The pharmacokinetics of MPA varies widely between and within patients and with time post-transplant [1–3]. The entero-hepatic cycling of MPAG may partly explain some of this variation. Further, it has been shown that the pharmacokinetics of MPA is influenced by co-administration of calcineurin inhibitors [4,5].

MPA is an acidic drug that is more highly bound to albumin (97–98%) than MPAG is bound to albumin (≈82%) [1]. It has been shown that the free fraction of MPA is constant

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doi:10.1016/j.jchromb.2003.10.033

in stable renal transplant patients with preserved renal function. However, in renal failure a two- to three-fold increase in MPA free fraction has been reported [6,7]. Elevated free concentrations of MPA have been associated with severe infections and leucopenia [8,9]. Such changes in free MPA concentrations may lead to an altered efficacy or toxicity profile.

Whilst MPAG is pharmacologically inactive, it has significant displacement effects on the binding of MPA to human serum albumin at clinically relevant concentrations [6,10]. MPAG concentrations are elevated in kidney transplant patients with delayed graft function [3,11]. In vitro studies with uremic and normal serum have shown the displacement effects of MPAG are additive to those caused by uremia alone [7]. Given the potential for MPAG to influence MPA binding, it is likely to be important to measure both the total and free concentrations.

Several techniques are available for the isolation of free drug from plasma; these include equilibrium dialysis, microdialysis, ultracentrifugation and ultrafiltration. Ultrafiltration, a simple and fast technique, has been validated for the isolation of free MPA [10]. Shipkova et al. [12] used this ultrafiltration process combined with HPLC-UV to measure free MPA concentrations in human plasma. We have previously developed HPLC assays to measure total concentrations of MPA and MPAG using ultraviolet detection [13] and free concentrations of MPA by mass spectrometric detection [14]. The aim of this study was to develop an improved method for free MPA and a new method for free MPAG.

2. Experimental

2.1. Chemicals and materials

All solvents were HPLC-grade and all reagents analytical-reagent grade. HPLC quality water was prepared using a Millipore Milli-Q Purification system (Millipore, Milford, MA, USA). Isolute C_{18} solid-phase extraction cartridges (200 mg, 3 ml) were obtained from IST (Mid Glamorgan, UK) and C_{18} cartridges (100 mg, 1 ml) from Waters (Milford, MA, USA). The Centrifree Micropartition System consisting of a 1 ml reservoir and a 30 000 Da MW cutoff membrane were used for ultrafiltration (Amicon, Danvers, MA, USA) of plasma samples.

2.2. Mycophenolic acid

2.2.1. Materials

Mycophenolic acid was a gift from F. Hoffman-La Roche Ltd. (Basel, Switzerland). The internal standard, indomethacin was purchased from ICN Biomedicals Inc. (Aurora, OH, USA). A working stock of internal standard (500 μg/l) was prepared in methanol. Standards (1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 100 and 200 μg/l) and quality control samples (7.5, 40.0 and 150 μg/l) were not prepared in plasma ultrafiltrate due to the very large volumes of ultrafiltrate required. Instead all standards and quality control samples were prepared in sodium chloride solution (9 g/l, pH 7.4), which has previously been validated as a suitable matrix for such samples [12].

2.2.2. HPLC-tandem mass spectrometry apparatus and conditions

The chromatographic separation was performed using an Agilent 110 Series system (Agilent Technologies, Palo Alto, CA, USA) with an Allure pentafluorophenylpropyl (PFPP) column (50 mm × 2.1 mm i.d., 5 μm, Restek, Bellefonte, PA, USA), maintained at ambient temperature. The mobile phase consisted of 75% methanol and 25% ammonium formate (40 mM, pH 3.3). The flow-rate was 0.2 ml/min with a post-column split (1:7) into the mass spectrometer ion source.

Mass spectrometric detection was performed on an API III triple quadrupole instrument (PE-Sciex, Thornhill, Toronto, Canada) using electrospray ionisation. Mycophenolic acid and internal standard were detected using selected reactant monitoring (*m/z* 338.2 → 206.9 and *m/z* 375.0 → 139.0, respectively). The energy for collision-induced fragmentation was −21.0 V. The electrospray ionisation interface was operated in positive ionisation mode with the orifice potential set to 40.0 V. The nebulizer and auxiliary gas flows were maintained at 0.6 l/min, respectively. Data was acquired with a dwell time of 600 ms, a pause time of 5 ms and a scan rate of 0.55/s.

2.2.3. Sample preparation

Ultrafiltration conditions were based on the method described by Nowak and Shaw [10]. Plasma samples (500 μl) were placed in sealed ultrafiltration tubes and centrifuged at 3000 × *g* in a Beckman fixed rotor centrifuge (20 min, 20 °C). It was confirmed that a freeze–thaw cycle did not alter MPA protein binding. The standard and quality control samples (100 μl) in sodium chloride solution, and patient ultrafiltrate samples (100 μl) were added to internal standard working stock (50 μl) and 0.05 M hydrochloric acid (400 μl) in glass culture tubes. Samples were vortex-mixed (1 min) and centrifuged (1 min, 850 g). The mixtures were applied to 200 mg C_{18} solid-phase extraction cartridges (isolute) that had been pre-conditioned with methanol (2 ml) and water (2 ml). The loaded cartridges were washed sequentially with water (2 ml) and 50% methanol/0.02 M ammonium formate buffer, pH 3.0 (1 ml). The washed cartridges were placed under full vacuum for 15 min. The analytes were eluted with methanol (2 ml) and all samples were vortexed before removing the solvent under a stream of air (45 °C). The residue was dissolved in mobile phase (100 μl). An aliquot of the mixture (20 μl) was injected on column.

2.2.4. Assay validation

The specificity of the method was evaluated by screening 10 ultrafiltrate samples from transplant patients not re-

ceiving mycophenolate mofetil therapy. The linearity of the free MPA assay was assessed over the concentration range 1.0–200 μg/l using a weighted $1/x^2$ regression model. The inter-day imprecision and accuracy of the assay with regard to the standards was determined from the back-calculated results of the linearity study over 8 days. The intra-day imprecision and accuracy was determined by assaying the quality control samples, as well as the lowest standard, replicated four times on day 1. To determine absolute assay recovery blank ultrafiltrate ($n = 4$) spiked with either high or low concentrations of mycophenolic acid with internal standard was extracted using solid-phase extraction. Additional blank ultrafiltrate was extracted ($n = 4$) and the eluate spiked with the same high or low concentrations of mycophenolic acid with internal standard. Peak areas of extracted ultrafiltrate spiked before and after extraction were then compared.

2.3. Free MPAG

2.3.1. Materials

MPAG and internal standard (carboxy butoxy ether mycophenolic acid) were obtained from Roche Bioscience (Palo Alto, CA, USA). A working stock of internal standard was made (33 mg/l) in methanol. Standards (1.0, 2.5, 5.0, 10.0, 25.0, 50.0 mg/l) and quality control samples (5.0, 20.0 and 45 mg/l) were prepared in sodium chloride solution (9 g/l, pH 7.4).

2.3.2. HPLC apparatus and conditions

The liquid chromatographic system consisted of a 616 pump with 600S controller (Waters), an IS200 autosampler (Perkin-Elmer, Danbury, CT, USA), a 484 tunable UV absorbance detector, a Nova-Pak C_{18} column (150 mm × 3.9 mm i.d., 4 μm, ambient temperature) and Maxima software (Waters). MPAG and its internal standard were separated using a binary gradient (20 min cycle time) at a flow rate of 2 ml/min. Initial gradient conditions were 100% mobile phase A (20% acetonitrile/orthophosphoric acid (0.05%)). After sample injection, the initial composition was held for 6 min, followed by a 9 min linear change to 100% mobile phase B (28% acetonitrile/orthophosphoric acid (0.05%)). The HPLC column was re-equilibrated for 5 min with mobile phase A, prior to injection of the next sample.

2.3.3. Sample preparation

Ultrafiltration conditions were based on the method described by Nowak and Shaw [10]. Plasma samples (500 μl) were placed in sealed ultrafiltration tubes and centrifuged at 3000 × *g* in a Beckman fixed rotor centrifuge (20 min, 20 °C). The patient ultrafiltrate, standard and quality control samples (100 μl) were added to internal standard working stock (50 μl) and 0.05 M hydrochloric acid (400 μl) in glass culture tubes. Samples were vortex-mixed (1 min) and centrifuged (1 min, 850 × *g*). The mixtures were applied to 100 mg C_{18} solid-phase extraction cartridges (Waters) that had been pre-conditioned with methanol (1 ml) and water (1 ml). The loaded cartridges were washed with 20% methanol/0.05 M potassium phosphate buffer, pH 3.0 (1 ml). The washed cartridges were placed under full vacuum for 5 min. The analytes were eluted with 80% methanol/0.05 M potassium phosphate buffer, pH 3.0 (1 ml). An aliquot of the mixture (50 μl) was injected on column.

2.3.4. Assay validation

The specificity of the free MPAG assay was evaluated by screening nine ultrafiltrate samples from transplant patients not receiving mycophenolate mofetil therapy. The linearity of the method was assessed over the concentration range (1.0–50.0 mg/l) using weighted $1/x^2$ regression models. The inter-day imprecision and accuracy of the assay with regard to the standards was determined from back calculating the results of the linearity study over 7 days. The intra-day imprecision and accuracy was determined by assaying the quality controls as well as the lowest standard, in replicates of four on day 1. To determine absolute assay recovery blank ultrafiltrate ($n = 4$) spiked with either high or low concentrations of mycophenolic acid with internal standard was extracted using solid-phase extraction. Additional blank ultrafiltrate was extracted ($n = 4$) and the eluate spiked with the same high or low concentrations of mycophenolic acid with internal standard. Peak areas of extracted ultrafiltrate spiked before and after extraction were then compared.

2.4. Free MPA/MPAG pharmacokinetic profile

The pharmacokinetics of free MPA and MPAG were investigated in a 68-year-old female renal transplant patient who was receiving oral dosing of MMF (CellCept®, 1 g bid). Primary immunosuppressive therapy was cyclosporin (Neoral®). Cyclosporin dose was adjusted to obtain a target 2 h post-dose cyclosporin concentration of 1300 to 1700 μg/l. Blood samples were collected on day 5 post-transplant, at times pre-dose and 1, 3 and 6 h after the dose, into EDTA tubes. Plasma was isolated by centrifugation at 800 × *g* for 15 min. Plasma samples were stored at −80 °C until day of analysis. Measurement of total MPA and MPAG in plasma was performed according to our previously reported method [13]. The areas under the concentration–time curve from 0 to 6 h ($AUC_{0\text{-}6}$) were calculated for free and total mycophenolic acid and its glucuronide using the trapezoidal rule. The percentage of unbound (free) mycophenolic acid was calculated by dividing the $AUC_{0\text{-}6}$ free by the $AUC_{0\text{-}6}$ total (×100%).

3. Results

3.1. Free MPA HPLC-MS/MS assay

Assay selectivity was demonstrated by the absence of interfering peaks at the retention times of MPA and its inter-

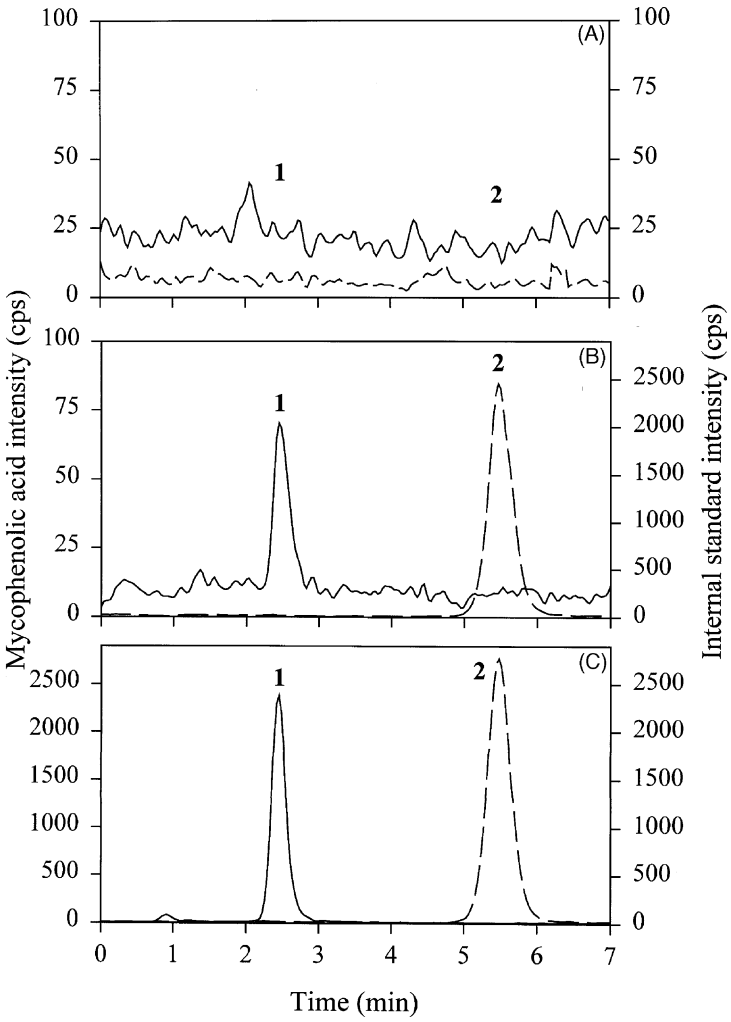

Fig. 1. Representative chromatograms for free MPA and its internal standard (IS) in blank plasma (A) compared to the lowest standard (1.0 μg/l; B) and a patient sample (51.9 μg/l; C). Retention times of MPA and internal standard are 2.3 and 5.3 min, respectively.

nal standard in 10 blood samples from transplant recipients not receiving mycophenolate mofetil. The retention times of MPA and the internal standard, indomethacin, were 2.3 and 5.3 min, respectively. The total chromatographic analysis time was 7 min. Fig. 1 shows typical chromatograms for free MPA and internal standard in blank plasma (A) compared to the lowest standard (1.0 μg/l; B) and a patient sample (51.9 μg/l; C). The assay was linear for MPA over the range 1.0–200 μg/l ($r^2 > 0.999$, $n = 8$). The lower limit of quantification (LLOQ) was 1.0 μg/l, with values of imprecision (9.3%) and accuracy (105.9%) acceptable under the proposed guidelines of Shah et al. [15]. The intra- and inter-day accuracy of the method determined using both quality controls and the lowest standard was 96.8–106.0% and 94.3–104.2%, respectively. The imprecision of the method determined using both quality controls and the lowest standard was <9.3 and <9.4% (expressed in terms of intra- and inter-day coefficients of variation), re-

Table 1
Accuracy and imprecision of the free MPA HPLC-MS/MS assay as measured using quality controls

Weighed-in concentration (μg/l)	Mean (μg/l)	Accuracy (%)		Imprecision (%)	
		Intra-day ($n = 4$)	Inter-day ($n = 8$)	Intra-day ($n = 4$)	Inter-day ($n = 8$)
7.5	7.3	97.1	96.7	2.2	9.4
40	41.7	101.2	104.2	2.2	6.8
150	141.4	96.8	94.3	2.6	8.0

Accuracy, mean concentration of measurements divided by the weighed-in concentration and expressed as a percentage; imprecision, co-efficient of variation of the measurements expressed as a percentage.

spectively. The imprecision and accuracy of the assay for the quality control data only is summarized in Table 1. The mean absolute recovery ($n = 4$) of mycophenolic acid, assessed at concentrations of 7.5 and 150 μg/l, was 81.5±1.9% and 85.2 ± 3.8%, respectively. The mean recovery of internal standard at these respective concentrations of MPA was 88.2 ± 2.4% and 86.8 ± 4.4%.

3.2. Free MPAG HPLC-UV assay

The method was specific for MPAG, as shown by lack of interference at the retention time of the two analytes in nine blood samples from transplant recipients not receiving mycophenolate mofetil. The retention times of MPAG and the internal standard, carboxy butoxy ether MPA, were 4.6 and 14.8 min, respectively. Fig. 2 shows representative chromatograms for free MPAG and internal standard in (A) blank plasma, (B) the lowest standard (1.0 mg/l) and (C) a patient sample (45.8 mg/l). The assay was linear over the range 1.0–50.0 mg/l ($y = 3.13(\pm1.30)x + 15.03(\pm0.88)$, $r = 0.992$, $n = 7$). The lower limit of quantification (LLOQ) was defined according to the performance data, as the standard at 2.5 mg/l. The intra- and inter-day accuracy of the method determined using both quality controls and the lowest standard was 90.3–106.0% and 100.2–108.0%, respectively. The imprecision of the method determined using both quality controls and the lowest standard was <6.5 and <7.5% (expressed in terms of intra- and inter-day coefficients of variation). The imprecision and accuracy of the assay for the quality control data only is summarized in Table 2. The mean absolute recovery ($n = 4$) of mycophenolic acid glucuronide, assessed at concentrations of 5.0 and 45.0 mg/l, was 76.8 ± 4.3% and 76.6 ± 1.6%, respectively. Recovery of internal standard at these respective concentrations of MPAG was 63.7 ± 2.5% and 59.7 ± 1.8%.

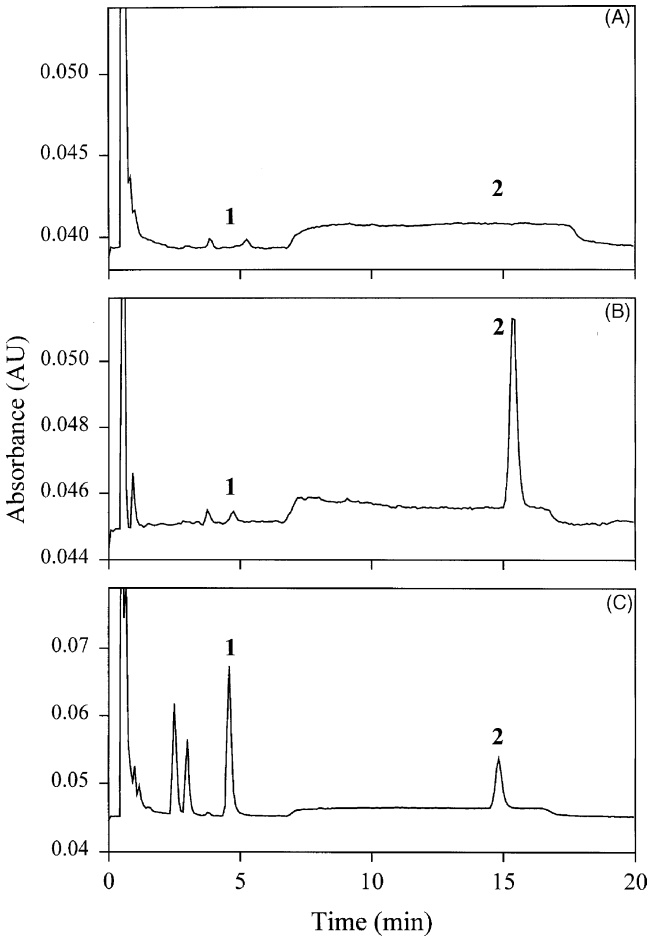

Fig. 2. Representative chromatograms for free MPAG and its internal standard (IS) in blank plasma (A) compared to the lowest standard (1.0 mg/l; B) and a patient sample (45.8 mg/l; C). Retention times of MPAG and internal standard are 4.6 and 14.8 min, respectively.

3.3. Free MPA/MPAG pharmacokinetic study

The concentration–time profile for free versus total MPA and MPAG concentrations in blood from a single renal patient on MMF therapy is shown in Fig. 3. The maximum concentration of free MPA measured using a 6.0 h limited sampling regime was at 3.0 h (430.5 μg/l). The maximum free MPAG concentration was also at 3.0 h and was nearly 200 fold higher than for free MPA at 76.6 mg/l. Total MPA and MPAG concentrations ranged between 0.7 and 5.1 mg/l, and between 136 and 190 mg/l, respectively. Calculated using the linear trapezoidal rule, the MPA $AUC_{0–6\,h}$ was 1508.3 μg h/l and MPAG $AUC_{0–6\,h}$ was 349.8 mg h/l. The percentage of unbound, or free MPA and MPAG for this patient was 7.3 and 34.9% respectively.

Table 2
Accuracy and imprecision of the free MPAG HPLC-UV assay as measured using quality controls

Weighed-in concentration (mg/l)	Mean (mg/l)	Accuracy (%)		Imprecision (%)	
		Intra-day ($n = 4$)	Inter-day ($n = 8$)	Intra-day ($n = 4$)	Inter-day ($n = 8$)
5.0	5.2	102.2	103.7	5.6	7.2
20	21.6	106.1	108.0	2.1	6.5
45	45.5	93.3	101.2	1.7	6.0

Accuracy, mean concentration of measurements divided by the weighed-in concentration and expressed as a percentage; imprecision, co-efficient of variation of measurements expressed as a percentage.

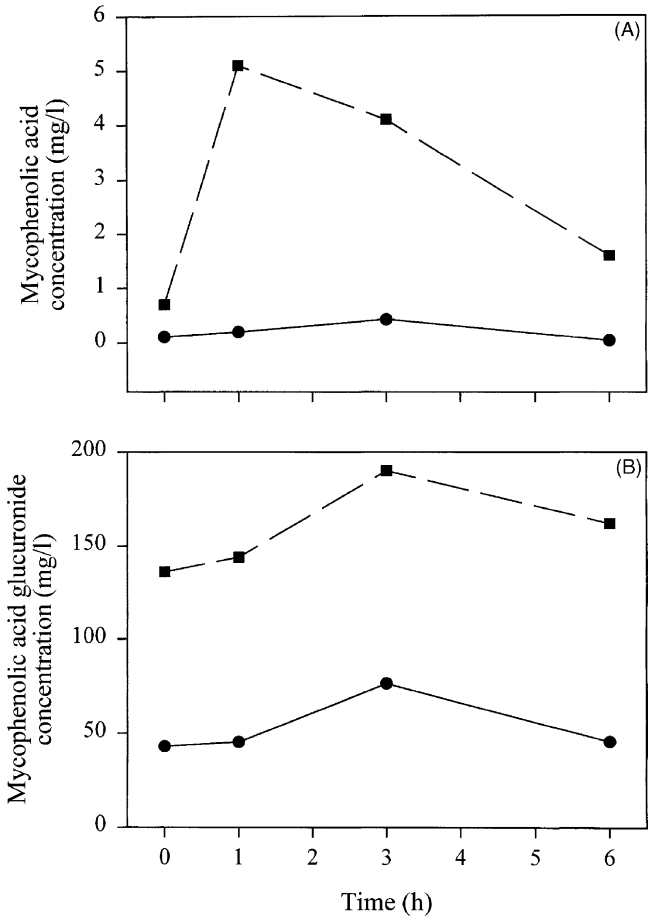

Fig. 3. Six hour pharmacokinetic profiles of mycophenolic acid (A) and mycophenolic acid glucuronide (B) from a 68-year-old female renal transplant recipient receiving chronic oral dosing of MMF (CellCept®, 1 g bid) co-administered with cyclosporin (the solid lines and broken lines indicate free and total concentrations, respectively).

4. Discussion

Although free MPA is the active component of MMF therapy, limited data are available on the relationship between free MPA and clinical outcomes or adverse events [16]. A relationship between total MPA AUC and rejection has been demonstrated [17] and an association between free MPA concentrations and leucopenia has been shown in paediatric renal transplant recipients [9]. Further, the role MPAG influences protein binding of MPA is not totally understood.

One limiting factor in the investigation of free MPA pharmacokinetics has been the lack of a reliable assay that can measure the drug to low concentrations using a small sample volume (<200 μl of ultrafiltrate). The determination of free drug concentration using HPLC-MS has recently been reviewed and although there are limited published methods, there is significant potential for HPLC-MS, in particular HPLC-MS/MS to facilitate free drug investigation [18]. Previously we reported use of HPLC-MS/MS for the quantification of free MPA using C_{18} column chromatography with atmospheric pressure chemical ionisation that enabled the measurement of MPA concentrations from 2.5 to 200 μg/l [14]. Shipkova and colleagues measured to a 10 μg/l limit of quantification (100 μl of ultrafiltrate) using an HPLC-UV method [12]. In developing the current method, increased sample throughput was sought together with increased assay sensitivity. Simultaneous measurement of free MPAG was also desirable. It was hoped that these improvements would provide a more suitable method for investigating free MPA and MPAG pharmacokinetics.

The current method described has two major modifications from our earlier work [14]. The ion source was changed from atmospheric pressure chemical ionisation to electrospray and a PFPP column was used for chromatography in place of the C_{18} column. These changes produced a five-fold increase in sensitivity. PFPP columns have only recently been used for HPLC-MS/MS assays [19]. These columns provide similar retention to C_{18} phase columns, but allow increased mobile phase organic strength, leading to increased ionisation efficiency and thus enhanced signal [20]. Needham et al. [19], for example, found the MS response for cocaine was a factor of 12 times greater on the PFPP phase (90% acetonitrile) than on C_{18} phase (12% acetonitrile). An added advantage of using the PFPP column was a reduction in sample analysis time to 7 min compared with our earlier 12 min method [14].

Indomethacin was the chosen internal standard for this free MPA HPLC-MS/MS assay instead of the more closely related compound (carboxy butoxy ether mycophenolic acid), used in HPLC-UV assays of total MPA [13]. The carboxy butoxy ether derivative of MPA was assessed during development of the HPLC-MS/MS assay, but provided unreliable results for quantitation, due to in-source degradation.

In the development of a simultaneous assay for MPA and MPAG, it was found that when MPAG eluted in the solvent front (void volume) results were unreliable (data not shown). This variability was likely due to ion suppression. To obtain retention on the PFPP column a large decrease in organic solvent strength was required, resulting in a significant loss of mass spectrometer response. Thus, it was decided to develop a separate HPLC-UV method for free MPAG. By minimizing sample volume requirements, the ultrafiltrate sample could be sub-divided to perform both free MPA and MPAG measurements from a single isolation procedure.

The measurement of free MPAG concentrations by HPLC-UV has not been previously reported. Jones et al. [13] resolved total plasma MPAG from interfering peaks using an isocratic separation. However, in developing this current method, there was between column variability in retention. Some columns provided insufficient resolution from these interfering peaks. Thus, a binary gradient was used to achieve the required separation. The increased resolution of MPAG from interfering peaks with the current method enabled the measurement of free MPAG concentrations from 2.5 to 50 mg/l using an ultrafiltrate volume of 100 μl.

The 6 h pharmacokinetic profile obtained from a renal transplant patient on MMF therapy is consistent with pub-

lished values for total MPA (1–7 mg/l) [1,2]. Total MPAG concentrations measured in this study were slightly higher than those previously reported (80–160 mg/l) which may be a result of mild renal impairment occurring acutely after transplant. Total MPAG concentrations greater than 100 mg/l have previously been associated with decreased MPA-albumin binding in vitro [10]. The patient's free fraction for MPA (7.3%) and MPAG (34.9%) was higher than healthy individuals (2 and 18%, respectively). This may reflect competitive displacement mechanisms via accumulation of MPAG in plasma. These results are consistent with those in adult kidney recipients with chronic renal dysfunction [7,11].

A novel method for measuring free MPAG has been developed, as well as an improved method for measuring free MPA. Measuring free concentrations of these analytes should provide a more complete understanding of the MPA pharmacokinetic variability observed in transplant populations. Further the HPLC-MS/MS method is suitable for therapeutic drug monitoring of free MPA.

Acknowledgements

National Health and Medical Research Council Project Grant number 210173 supported this study.

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