Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 846 (2007) 313–318
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Short communication

# Simple reversed-phase liquid chromatographic assay for simultaneous quantification of free mycophenolic acid and its glucuronide metabolite in human plasma

Wai-Ping Yau [a], Anantharaman Vathsala [b], Huei-Xin Lou [c], Shu Feng Zhou [a], Eli Chan [a,*]

[a] Department of Pharmacy, Faculty of Science, National University of Singapore, 18 Science Drive 4, Singapore 117543, Singapore
[b] Department of Renal Medicine, Singapore General Hospital, Outram Road, Singapore 169608, Singapore
[c] Department of Pharmacy, Singapore General Hospital, Outram Road, Singapore 169608, Singapore

Received 3 June 2006; accepted 27 July 2006
Available online 28 August 2006

**Abstract**

A reversed-phase HPLC-UV method, involving simple instrumental setup and mobile phase without ion-pairing reagent, was developed and validated for direct simultaneous quantification of free mycophenolic acid (MPA) and its major metabolite MPA-glucuronide (MPAG) in human plasma. Both free MPA and MPAG were isolated from plasma samples using ultrafiltration prior to analysis. Each chromatographic run was completed within 13 min. The optimized method showed good performance in terms of specificity, linearity ($r^2 = 0.9999$), sensitivity (limit of quantitation (LOQ): 0.005 mg/L for MPA; 1 mg/L for MPAG), and intra- and inter-day precision (R.S.D. < 7%). This assay was successfully applied to free MPA and MPAG measurements in clinical samples.


*Keywords:* Mycophenolic acid; Mycophenolic acid glucuronide; Reversed-phase HPLC; Free drug; Human plasma

## 1. Introduction

Mycophenolate mofetil (MMF) is an immunosuppressant to prevent organ rejection in transplant recipients. After oral administration, MMF is hydrolyzed to the active entity, mycophenolic acid (MPA), which is metabolized to its main inactive metabolite MPA-glucuronide (MPAG) that may be converted back to MPA via enterohepatic circulation [1,2]. In terms of protein binding, MPA and MPAG are around 97.5% and 82% bound to human serum albumin, respectively [1].

In a roundtable discussion, total MPA measurement has been proposed for therapeutic drug monitoring [3]. Since the immunosuppressive activity of MPA is dependent on its free concentration [4], free MPA would be a better indicator of drug exposure as compared to total MPA. Moreover, free MPA, but not total MPA, has been reported to correlate with the occurrence of hematological side effects, such as leukopenia [5,6], thrombocytopenia [5] and anemia [7], as well as infections [5,6]. All these findings thus support the measurement of free MPA. In addition, as the free fraction of MPA may be significantly altered by many clinical conditions that affect protein binding, such as hypoalbuminemia, uremia and hyperbilirubinemia, free MPA monitoring would be especially desirable in these situations [3]. It would also be of interest to measure free MPAG as increased MPAG in conditions like renal impairment or delayed graft function would compete with MPA for protein binding [3].

To date, validated assays have been reported for quantifying free MPA [8–16] or free MPAG [16] alone. Four assays for the simultaneous quantification of free MPA and MPAG have also been developed [17–20] but they suffer from certain drawbacks. These include the inconvenience of indirect measurement of MPAG after enzymatic hydrolysis to MPA [17], use of complicated and costly setup [18,20] and employment

* Corresponding author. Tel.: +65 6516 2932; fax: +65 6779 1554.
*E-mail address:* phaelic@nus.edu.sg (E. Chan).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.07.062

of time-consuming extraction procedure [19]. Hence, there is a need for a simple, economical yet efficient and effective assay for the simultaneous determination of free MPA and MPAG levels in plasma samples, to enable free MPA and MPAG monitoring to be easily carried out in clinical practice.

In the *in vitro* measurement of free drug concentration in plasma, the free drug must first be separated from the protein-bound drug by equilibrium dialysis, ultrafiltration or ultracentrifugation [21]. Of these, ultrafiltration is most preferred in the clinical setting due to its technical simplicity and rapidity [21].

We have previously developed an ion-pair HPLC-UV assay for the simultaneous determination of total MPA and MPAG [22] but it was not sensitive enough for free drug measurement. This paper thus aims to develop and validate a simple non-ion-pair HPLC-UV method with improved sensitivity for direct quantification of free MPA and MPAG simultaneously, using ultrafiltration as a simple and fast sample preparation procedure. The applicability of the developed assay for clinical pharmacokinetic studies will also be investigated.

## 2. Experimental

### 2.1. Chemicals and reagents

MPA was obtained from Fluka Chemie (Buchs, Switzerland). MPAG and the carboxybutoxy ether of MPA (MPAC) were generous gifts from Roche Bioscience (Palo Alto, CA, USA). AR-grade phosphoric acid 85% and HPLC-grade acetonitrile were purchased from Mallinckrodt Baker (Paris, KY, USA). Pooled blank human plasma was obtained from the blood bank of the National University Hospital, Singapore. Milli-Q (18 MΩ) water was generated by a Milli-Q RG Millipore Water Purification System (Millipore, SA, Molsheim, France).

### 2.2. Ultrafiltration conditions

Vivaspin-2 ultrafiltration devices (Vivascience Inc., Hannover, Germany), each consisting of a membrane with 5000 Da molecular weight cutoff (MWCO), were used. Plasma samples (500 μL) were added to each device and centrifuged at 3000 g for 20 min at 37 °C using an Avanti™ J-25 Centrifuge (Beckman Instruments, CA, USA) to obtain ultrafiltrates. Protein leakage through the membrane was checked by determining the protein concentration in the ultrafiltrates using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). The possibility of any non-specific binding of MPA and MPAG to the ultrafiltration device was assessed by ultrafiltration of phosphate buffered saline samples spiked separately with MPA and MPAG, and determining the concentrations in the pre- and post-ultrafiltered specimens.

### 2.3. Preparation of calibration standards

Standard stock solutions of MPA (100 mg/L), MPAG (5000 mg/L) and the internal standard (I.S.), MPAC (1000 mg/L), were prepared in methanol and stored at −20 °C. Working solutions of MPA, MPAG and MPAC in methanol–water (8:2, v/v) were prepared by appropriate dilution of the stock solutions. For the calibration standards, aliquots of 150 μL of blank ultrafiltrate from pooled blank human plasma were spiked with 10 μL each of the MPA, MPAG and MPAC working solutions to yield spiked ultrafiltrate concentrations from 0.005 mg/L to 2 mg/L for MPA, from 1 mg/L to 150 mg/L for MPAG, and 15 mg/L for MPAC.

### 2.4. Instrumentation and chromatographic conditions

A Shimadzu integrated HPLC system LC-2010A liquid chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a UV detector, autosampler, column oven and data processing software (Shimadzu Class-VP software version 6.10) was used. All chromatographic separations were performed using an Atlantis™ $dC_{18}$ analytical column (150 mm × 4.6 mm i.d., particle size 5 μm) (Waters Corporation, Milford, MA, USA), connected with an Atlantis™ $dC_{18}$ guard column (20 mm × 4.6 mm i.d., particle size 5 μm) (Waters).

The mobile phase consisted of 0.05% (v/v) aqueous phosphoric acid–acetonitrile (60:40, v/v), mixed on-line and delivered at a flow rate of 1 mL/min. The column oven temperature was maintained at 30 °C. Each sample was injected at 100 μL for an analysis run of 13 min and UV detection was set at 304 nm.

### 2.5. Specificity

Potential chromatographic interferences by drugs commonly co-administered with MMF by transplant patients were evaluated. The UV spectra of these drugs were checked from Clarke's Analysis of Drugs and Poisons [23]. The drugs that do not exhibit UV absorbance at 304 nm would not interfere with the assay. For those that exhibit UV absorbance at 304 nm, each of these drugs was subject to HPLC analysis to assess for the presence of any potential interfering peaks.

### 2.6. Clinical samples for pharmacokinetics application

The applicability of this assay for pharmacokinetics studies was assessed on a stable renal transplant patient (male, Chinese, age: 46 years) receiving 500 mg MMF (CellCept®) twice daily for more than 3 months. The study was approved by the Ethics Committee of Singapore General Hospital and written informed consent was obtained from the patient. Blood samples were collected, processed and treated according to our previous method [22]. For free drug quantification, 500 μL of thawed plasma from each sample was incubated in a shaking water bath at 37 °C for 1 h and subject to ultrafiltration. The ultrafiltrate collected (150 μL) was treated as above for the calibration standards, except that 20 μL of methanol–water (8:2, v/v) was spiked in place of 10 μL each of MPA and MPAG working solutions. For total drug quantification, sample preparation and analysis were based on our published method [22]. The free and total concentrations of MPA and MPAG in the plasma samples were calculated by reference to calibration curves generated from calibration standards analyzed along with these samples.

## 3. Results and discussion

### 3.1. Method development

#### 3.1.1. Selection of the analytical column

The use of the Atlantis™ $dC_{18}$ analytical column, that is designed to provide enhanced polar compound retention without retaining non-polar compounds excessively, rendered it possible for simultaneous analysis of both free MPA and MPAG without the need of an ion-pairing agent in the mobile phase. This simplified the mobile phase preparation which is an advantage over our previous method [22] and that by Aresta et al. [19]. In addition, improved sensitivity was attained with the use of this analytical column as sharper and taller peaks were achieved as compared

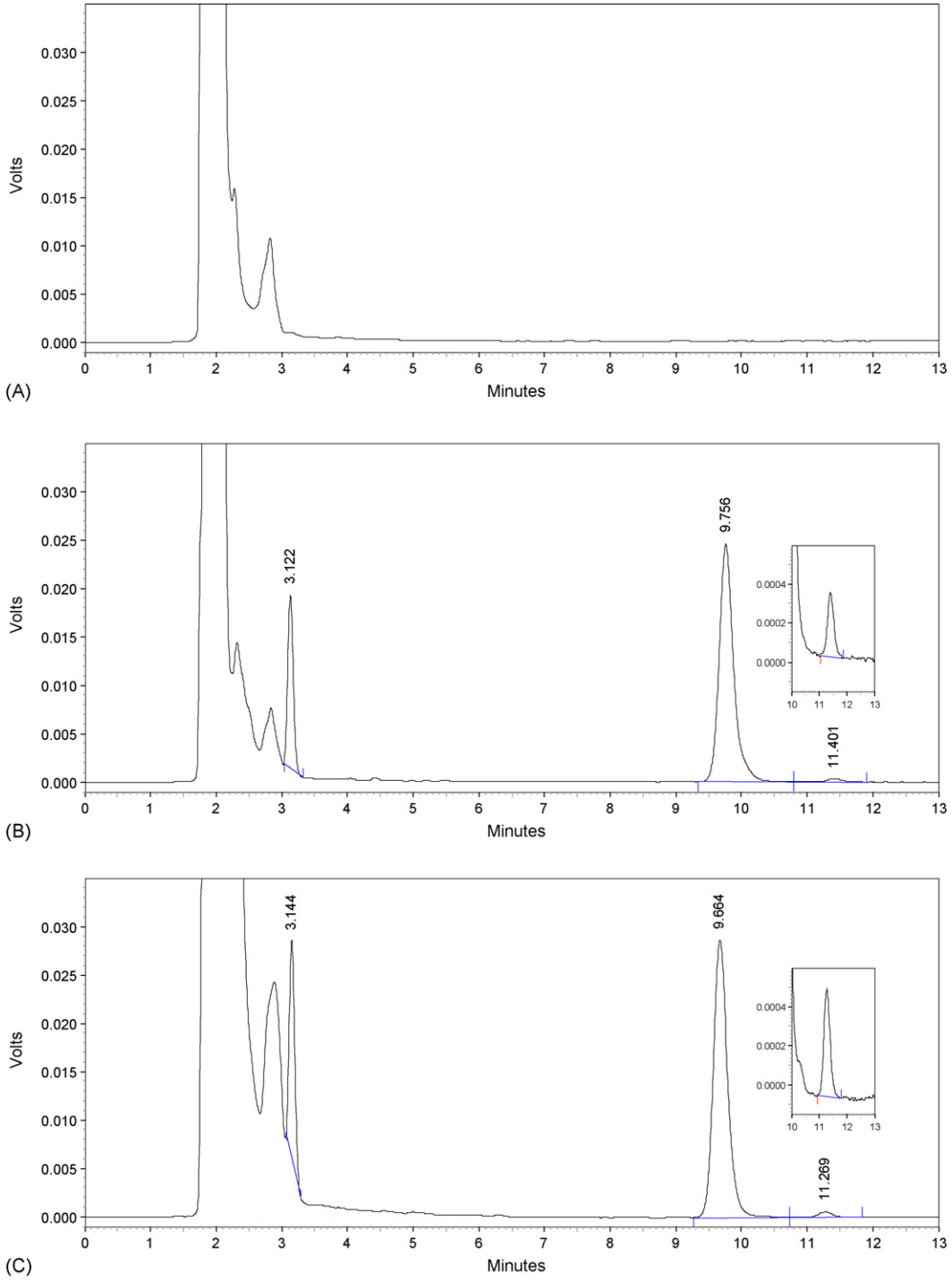


Fig. 1. Representative chromatograms showing the simultaneous analysis of free MPA and MPAG in human plasma: (A) blank ultrafiltrate; (B) blank ultrafiltrate spiked with MPA (0.05 mg/L), MPAG (10 mg/L) and MPAC (15 mg/L); (C) ultrafiltrate from the plasma sample of a renal transplant patient under immunosuppressive therapy with MMF obtained 0.5 h after MMF administration (free MPA: 0.0844 mg/L, free MPAG: 13.2 mg/L). The insets in (B) and (C) show the respective MPA peaks on a different absorbance scale. Retention times: MPAG ~3.1 min, MPAC ~9.7 min, MPA ~11.3 min.

with our reported ion-pair HPLC method using an XTerra™ $RP_{18}$ analytical column [22].

### 3.1.2. Sample preparation by ultrafiltration

The published methods for free MPA and free MPAG determination employed ultrafiltration devices with membrane MWCO of 10,000 Da [11,20] or 30,000 Da [9,13–16,19]. The Vivaspin-2 ultrafiltration device (MWCO: 5000 Da) was used in this study to improve protein removal. This device was tested to show insignificant non-specific binding of MPA and MPAG, and no protein leakage through the membrane. This allowed for the direct injection of protein-free ultrafiltrates into the HPLC for analysis without further extraction procedures.

## 3.2. Optimal conditions and assay validation

Under the fully optimized chromatographic conditions, representative chromatograms are presented in Fig. 1. The MPAG, MPAC and MPA peaks were well-resolved and no interfering peaks from endogenous plasma substances in blank ultrafiltrate (Fig. 1A) were observed. The relative peak area (analyte to I.S. peak area ratio) of MPA and MPAG were used for quantitative computations.

### 3.2.1. Specificity

Commonly administered concomitant drugs (Table 1) were evaluated to show no interference to the MPAG, MPAC and MPA peaks.

### 3.2.2. Linearity

Calibration curves were constructed by non-weighted least-squares linear regression of relative peak areas versus concentrations spiked to drug-free ultrafiltrate samples. Linearity was assessed based on the coefficient of determination (i.e. $r^2$) and visual inspection of the residual plots of the data points. Each calibration concentration was assayed in triplicates. Eight-point calibration curves were linear over the range of 0.005–2 mg/L for free MPA ($y = 0.000227x + 0.003392$, $r^2 = 0.9999$) and over the range of 1–150 mg/L for free MPAG ($y = 0.033042x - 0.010085$, $r^2 = 0.9999$).

### 3.2.3. Limits of detection and quantitation

The limit of detection (LOD), defined as the analyte concentration with a signal-to-noise ratio of three, were 0.0015 mg/L and 0.3 mg/L, for free MPA and MPAG, respectively. The limit of quantitation (LOQ), determined as the lowest point on the calibration curve that could be analyzed within 20% of the nominal value, were 0.005 mg/L and 1 mg/L, for free MPA and MPAG, respectively. The sensitivity of this method was much improved over our previous assay by more than or at least ten times [22]. This improvement was attained as a result of a five-fold increase in sample injection volume, the omission of a 1:1 addition of acetonitrile to plasma samples for protein precipitation, as well as the use of an Atlantis™ $dC_{18}$ analytical column in a non-ion-pair HPLC method instead of an XTerra™ $RP_{18}$ analytical column in an ion-pair HPLC method [22].

Table 1
Drugs that did not show interferences to MPAG, MPAC and MPA peaks under the optimized chromatographic conditions

| |
|---|
| Immunosuppressives |
| Cyclosporine |
| Prednisolone |
| Tacrolimus |
| Cardiac drugs |
| Amlodipine |
| Atenolol |
| Aspirin |
| Bezafibrate |
| Captopril |
| Diltiazem |
| Dipyridamole |
| Enalapril |
| Frusemide |
| Hydralazine |
| Imidapril |
| Indapamide |
| Isosorbide dinitrate |
| Losartan |
| Nifedipine |
| Pravastatin |
| Prazosin |
| Simvastatin |
| Warfarin |
| Anti-viral drugs |
| Aciclovir |
| Ganciclovir |
| Anti-diabetic drugs |
| Acarbose |
| Glipizide |
| Insulin |
| Metformin |
| Tolbutamide |
| Anti-gout drugs |
| Probenecid |
| Gastro-intestinal drugs |
| Famotidine |
| Omeprazole |
| Antibiotics |
| Amoxicillin |
| Sulfamethoxazole-trimethoprim |
| Others |
| Alpha-calcidol |
| Folic acid |

For free MPA quantification, the present method was more sensitive than the reported modified enzyme-multiplied immunoassay technique (EMIT) [8] and other HPLC-UV assays [9,17–19]. It was also more sensitive [20] or at least comparable [11] to HPLC methods using fluorescence detection. As compared to those using the more sophisticated MS [24] or tandem MS [13–16] detectors, the current method was found to be less sensitive.

As for free MPAG quantification, the current assay was comparable [18], if not better than [16,17,20] most of the published methods. It was not as sensitive as that by Aresta et al. [19] whereby the sample was subject to a rigorous sample

Table 2
Accuracy of the simultaneous free MPA and MPAG assay in human plasma

| | Drug concentration (mg/L) | | R.S.D. (%) | Mean absolute percentage error[b] (%) | Mean analytical recovery[c] (%) |
|---|---|---|---|---|---|
| | Spiked | Analyzed[a] (mean ± S.D.) | | | |
| MPA | 0.005 | 0.00451 ± 0.00046 | 10.2 | 9.80 | 90.2 |
| | 0.01 | 0.00937 ± 0.00033 | 3.61 | 6.30 | 93.7 |
| | 0.025 | 0.0247 ± 0.0023 | 9.21 | 1.16 | 98.8 |
| | 0.05 | 0.0517 ± 0.0037 | 7.18 | 3.48 | 103 |
| | 0.1 | 0.109 ± 0.002 | 1.41 | 8.95 | 109 |
| | 0.5 | 0.469 ± 0.033 | 7.05 | 6.23 | 93.8 |
| | 1 | 0.961 ± 0.041 | 4.24 | 3.87 | 96.1 |
| | 2 | 2.09 ± 0.12 | 5.62 | 4.45 | 104 |
| MPAG | 1 | 0.831 ± 0.063 | 7.60 | 17.0 | 83.0 |
| | 2.5 | 2.40 ± 0.23 | 9.70 | 4.00 | 96.0 |
| | 5 | 4.70 ± 0.11 | 2.28 | 6.00 | 94.0 |
| | 10 | 9.01 ± 0.48 | 5.33 | 9.90 | 90.1 |
| | 25 | 25.9 ± 1.3 | 4.90 | 3.48 | 103 |
| | 50 | 46.7 ± 3.2 | 6.88 | 6.64 | 93.4 |
| | 100 | 109 ± 8 | 7.02 | 9.18 | 109 |
| | 150 | 154 ± 10 | 6.37 | 2.57 | 103 |

[a] Average of three replicates.
[b] Defined as the absolute percentage of difference between the analyzed and the spiked concentration over that of the spiked value.
[c] Defined as the analyzed concentration expressed as a percentage of the actual spiked concentration.

extraction procedure to concentrate it five times prior to HPLC analysis.

Nonetheless, the present assay was simple and economical, and its sensitivity was sufficient for free MPA and MPAG quantification in clinical plasma samples.

### 3.2.4. Precision and accuracy

Assay precision was determined by triplicate analyses of ultrafiltrate samples at each of three spiked concentrations (MPA: 0.025 mg/L, 0.5 mg/L, 2 mg/L; MPAG: 5 mg/L, 50 mg/L, 150 mg/L) within the same day (i.e. intra-day precision) and over three consecutive days (i.e. inter-day precision). The intra- and inter-day precisions (R.S.D.) for both free MPA and MPAG were less than 7.0%.

The accuracy of the assay was assessed by evaluating the analytical recoveries and mean absolute percentage errors of free MPA and MPAG concentrations over the linearity ranges, with each concentration being assayed in triplicates. The results are presented in Table 2.

### 3.2.5. Stability

The stability of free MPA and MPAG has been established in literature. Plasma protein binding was reported to be unaltered after 14 days at 4 °C or at −20 °C with exposure to three freeze-thaw cycles for both MPA and MPAG [18], and even after 6 months at −20 °C with one freeze-thaw cycle for MPA [14]. Free MPA and MPAG in ultrafiltrate were reported to be stable for at least 24 h at room temperature, and also after three freeze-thaw cycles [20].

## 3.3. Clinical application

The 12-h pharmacokinetic profiles of free and total MPA and MPAG in plasma from an individual stable transplant patient are presented in Fig. 2. The calculated area under the plasma concentration–time curve at steady-state for total MPA and MPAG ($AUC_{ss,total}$) was 23.1 mg h/L and 663 mg h/L, respectively, while that for free MPA and MPAG ($AUC_{ss,free}$) was 0.409 mg h/L and 136 mg h/L, respectively. The percentage of free MPA or MPAG for this patient, calculated by the percent ratio of $AUC_{ss,free}$ to $AUC_{ss,total}$, was 1.77% and 20.5%, respectively.

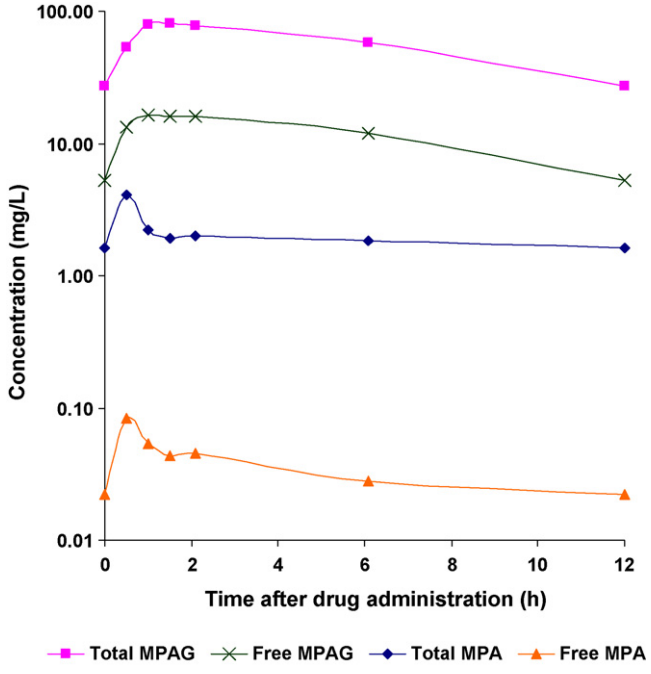


Fig. 2. Pharmacokinetic profiles of both free and total MPA and MPAG of an individual stable renal transplant patient under chronic immunosuppressive therapy, receiving 500 mg MMF (CellCept®) twice daily.

## 4. Conclusion

A simple non-ion-pair HPLC-UV method with improved sensitivity was developed for the simultaneous direct quantification of free MPA and MPAG in human plasma. Owing to its sensitivity, simplicity, rapidity and successful application in clinical pharmacokinetic studies, this validated assay could be potentially useful for therapeutic drug monitoring of free MPA and MPAG.

## Acknowledgements

This study was supported by the National University of Singapore Academic Research Grant (R-148-000-050-112). The authors thank Roche Bioscience for kindly providing the MPAG and MPAC.

## References

[1] R.E. Bullingham, A. Nicholls, M. Hale, Transplant. Proc. 28 (1996) 925.
[2] R.E. Bullingham, A.J. Nicholls, B.R. Kamm, Clin. Pharmacokinet. 34 (1998) 429.
[3] L.M. Shaw, D.W. Holt, M. Oellerich, B. Meiser, T. van Gelder, Ther. Drug Monit. 23 (2001) 305.
[4] I. Nowak, L.M. Shaw, Clin. Chem. 41 (1995) 1011.
[5] B.A. Atcheson, P.J. Taylor, D.W. Mudge, D.W. Johnson, C.M. Hawley, S.B. Campbell, N.M. Isbel, P.I. Pillans, S.E. Tett, Br. J. Clin. Pharmacol. 59 (2005) 271.
[6] L.T. Weber, M. Shipkova, V.W. Armstrong, N. Wagner, E. Schutz, O. Mehls, L.B. Zimmerhackl, M. Oellerich, B. Tonshoff, J. Am. Soc. Nephrol. 13 (2002) 759.
[7] D. Cattaneo, F. Gaspari, S. Ferrari, N. Stucchi, L. Del Priore, N. Perico, E. Gotti, G. Remuzzi, Clin. Transplant. 15 (2001) 402.
[8] P.E. Wallemacq, J. Konig, E. Henry de Frhan, N. Maton, J.P. Squifflet, R. Verbeeck, M. Mourad, Ther. Drug Monit. 23 (2001) 474.
[9] M. Shipkova, P.D. Niedmann, V.W. Armstrong, E. Schutz, E. Wieland, L.M. Shaw, M. Oellerich, Clin. Chem. 44 (1998) 1481.
[10] A. Jain, R. Venkataramanan, I.S. Hamad, S. Zuckerman, S. Zhang, J. Lever, V.S. Warty, J.J. Fung, J. Clin. Pharmacol. 41 (2001) 268.
[11] J. Shen, Z. Jiao, Y.Q. Yu, M. Zhang, M.K. Zhong, J. Chromatogr. B 817 (2005) 207.
[12] M. Korecka, D. Nikolic, R.B. van Breemen, L.M. Shaw, Clin. Chem. 45 (1999) 1047.
[13] C. Willis, P.J. Taylor, P. Salm, S.E. Tett, P.I. Pillans, J. Chromatogr. B 748 (2000) 151.
[14] F. Streit, M. Shipkova, V.W. Armstrong, M. Oellerich, Clin. Chem. 50 (2004) 152.
[15] C.G. Patel, A.E. Mendonza, F. Akhlaghi, O. Majid, A.K. Trull, T. Lee, D.W. Holt, J. Chromatogr. B 813 (2004) 287.
[16] B. Atcheson, P.J. Taylor, D.W. Mudge, D.W. Johnson, P.I. Pillans, S.E. Tett, J. Chromatogr. B 799 (2004) 157.
[17] J.O. Svensson, C. Brattstrom, J. Sawe, Ther. Drug Monit. 21 (1999) 322.
[18] R. Mandla, P.D. Line, K. Midtvedt, S. Bergan, Ther. Drug Monit. 25 (2003) 407.
[19] A. Aresta, F. Palmisano, C.G. Zambonin, P. Schena, G. Grandaliano, J. Chromatogr. B 810 (2004) 197.
[20] Z. Jiao, Y. Zhong, J. Shen, Y.-q. Yu, Chromatographia 62 (2005) 363.
[21] C.K. Svensson, M.N. Woodruff, J.G. Baxter, D. Lalka, Clin. Pharmacokinet. 11 (1986) 450.
[22] W.P. Yau, A. Vathsala, H.X. Lou, E. Chan, J. Chromatogr. B 805 (2004) 101.
[23] A.C. Moffat, M.D. Osselton, B. Widdop (Eds.), Clarke's Analysis of Drugs and Poisons: In Pharmaceuticals, Body Fluids and Postmortem Material, vol. 2, Pharmaceutical Press, London, 2004.
[24] M. Korecka, L.M. Shaw, Ther. Drug Monit. 23 (2001) 471.