Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 817 (2005) 207–213
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Quantification of total and free mycophenolic acid in human plasma by liquid chromatography with fluorescence detection

Jie Shen[a,d], Zheng Jiao[a,*], Yun-Qiu Yu[b], Ming Zhang[c], Ming-Kang Zhong[a]

[a] *Department of Pharmacy, Huashan Hospital, Fudan University, Shanghai 200040, PR China*
[b] *Department of Pharmaceutical Analysis, School of Pharmacy, Fudan University, Shanghai 200032, PR China*
[c] *Department of Nephrology, Huashan Hospital, Fudan University, Shanghai 200040, PR China*
[d] *Department of Pharmacy, Huadong Hospital, Shanghai 200040, PR China*

Received 8 September 2004; accepted 6 December 2004
Available online 28 December 2004

Abstract

A simple high-performance liquid chromatographic (HPLC) method was developed for the assay of total and free mycophenolic acid (MPA) in human plasma. Prior to analysis, total mycophenolic acid was extracted by protein precipitation and free drug was isolated from plasma samples using ultrafiltration. The extracts were injected onto a Kromasil C_8 column at 30 °C with excitation and emission wavelengths set at 342 and 425 nm, respectively. The mobile phase was consisted of acetonitrile-32 mM glycine buffer, pH 9.2 (20:80, v/v), at a flow rate of 1.0 ml/min. The method was found to be linear over the concentration range investigated, 0.05–40 mg/l for total mycophenolic acid ($r > 0.999$) and 5–1000 μg/l ($r > 0.99$) for free drug. The percentage error of the analytical method was below 10.9%. The intra- and inter-day reproducibility was adequate with the coefficients of variation of 8.28% or below. The run time were 4 and 6 min for free and total MPA, respectively. The method thus can be effectively applied to measure mycophenolic acid concentrations in clinical samples.

Keywords: Mycophenolic acid

1. Introduction

Mycophenolate mofetil (MMF), the 2-(4-morpholino) ethyl ester of mycophenolic acid (MPA), has been approved for the prophylaxis of acute graft rejection in solid organ transplantation. Following oral administration of MMF, MMF is rapidly hydrolyzed to MPA, which is the active metabolite. MPA exerts its immunosuppressive activity by decreasing guanine nucleotide levels in proliferating lymphocytes via noncompetitive, selective and reversible inhibition of inosine monophosphate dehydrogenase [1].

Considerable individual variability has been observed in healthy volunteers and renal allograft recipients [1,2]. Enterohepatic circulation, UDP-glucuronyltransferases, MPA free fraction, the effect of time posttransplantation, concomitant drugs were major factors postulated to be responsible for this variability but yet to be explored [1,3,4]. In addition, renal dysfunction and liver disease may also influence MPA pharmacokinetics [1,2].

MPA is extensively bound to serum albumin (97–99%). It was shown that the free concentration of MPA, rather than the total concentration, was associated with the immunosuppressive effect and an increased risk of MMF toxicity [5,6]. In general, the free MPA is constant in stable renal transplant patients with preserved renal function. However, it can be significantly increased in patients with uremia, renal insufficiency, liver disease, or other causes of hypoalbuminemia [6–8]. These data indicated that the measurement of free MPA appeared to be more appropriate in those patients considered at risk for MPA-related toxicity.

* Corresponding author. Tel.: +86 21 32120059; fax: +86 21 32120059.
E-mail address: jiaozhen@online.sh.cn (Z. Jiao).

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.12.005

Methods are available for assaying total MPA in biological fluids using high-performance liquid chromatographic (HPLC) [9–22] and Emit assay [23,24]. It has been suggested that the antibody used in the Emit assay showed cross-reactivity with MPA glucuronide metabolite, thereby the measurements for Emit assay were higher than those determined by HPLC. For HPLC quantification, the methods coupled with UV detection [9–17] and fluorescence detection [18] involved low sensitivity, lengthy run time, and time-consuming sample pretreatment which may not be suitable for MPA routine monitoring.

In the development of free MPA method, several papers based on the utilization of ultrafiltration were described. Shipkova et al. [17] used ultrafiltration process combined with UV detection at 215 nm to measure free MPA concentration in human plasma. The limit of detection was 5 μg/l but the volume of injection was up to 100 μl. LC–MS methods involved solid-phase extraction [19,20] or a column-switching technique [21] after ultrafiltration and the applicability of these methods are limited as the equipment is not widely available. In addition, the automated sequential trace enrichment of a dialysated (ASTED) system has been applied to measure free MPA [22]. However, many laboratories with limited resources are unlikely to have access to special techniques and complicated procedures. So a simpler assay method is highly expected.

The present study described an alternative method for the determination of total and free MPA in a small volume of human plasma using fluorescence detection. The main features of this method were an improved sensitivity, simpler pretreatment, and the shorter run time for assaying total and free MPA. This method was validated and used for the quantification of both total and free MPA obtained from renal transplant recipients.

2. Experimental

2.1. Chemicals and reagents

MPA was obtained from Fluka Chemie Corporation (purity: > 98%, Buchs, Switzerland). Naproxen, used as internal standard (I.S.) was kindly provided by Shanghai institute for drug control (purity: 99.5%, Shanghai, PR China). Acetonitrile and methanol were HPLC grade from Burdick&Jackson Corporation (Muskegon, MA, USA). The water was filtered through the Millipore Milli-Q system (Milford, MA, USA). All other chemicals and solvents used were of analytical grade. The stock solution of MPA at a concentration of 20 g/l was prepared in methanol and diluted to the desired concentrations in methanol for the working solutions. The stock solution of I.S. as 1 g/l in methanol was diluted to 100 mg/l in methanol for the working solution. All stock solutions and working solutions were stored at 4 °C.

2.2. Preparation of assay standard sample

2.2.1. Total MPA

MPA standard samples (0.05, 0.2, 1.0, 10, 25, 40 mg/l) were prepared by spiking control human plasma with appropriate volumes of the working solutions prepared mentioned above. Quality control (QC) samples (0.05, 0.1, 15 and 30 mg/l) were independently prepared in the same manner. All standards and QC samples were stored at −20 °C until analysis.

2.2.2. Free MPA

Standards (5, 10, 100, 400, 700, 1000 μg/l) and QC samples (5, 8, 500, 800 μg/l) were not prepared in plasma ultrafiltrate due to the very large volumes of ultrafiltrate required. Instead all standards and QC 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 [17,19–21].

2.3. Extraction procedure

2.3.1. Total MPA

A 100 μl of standard, QC, or patient plasma samples was transferred into 1.5 ml polypropylene tubes and 300 μl methanol containing 100 mg/l of naproxen was added to each tube and this solution was vortexed for 30 s. The mixture was then centrifuged for 10 min (10,000 × *g* at 4 °C). A 20 μl aliquot of the clear supernatant was injected into the HPLC system for quantitation.

2.3.2. Free MPA

Ultrafiltration has been validated for isolation of free MPA as it offered significant advantages of simple commercially available kits and lack of dilution effects [17,19–21,25]. So separation of free MPA was done by ultrafiltration modified from the method described by Nowak and Shaw [25]. The disposable centrifree cartridges (Millipore, MA, USA) consisted of a sample reservoir containing 10,000 Da MW cutoff membrane, a retentate vial and a filtrate vial. Plasma samples (500 μl) were placed in sealed ultrafiltration tubes and centrifuged at 10,000 × *g* in a Beckman fixed rotor centrifuge (40 min, 25 °C) to achieve 200 μl ultrafiltrate. The ultrafiltrate (20 μl) was directly injected on column.

The absorption of MPA on the ultrafiltration system was studied at low (0.1 mg/l), median (16 mg/l) and high (32 mg/l) concentration of MPA in the sodium chloride solutions (9 g/l, pH 7.4). The aqueous solutions were ultrafiltered according to the described techniques. MPA concentrations were measured in the solution (Cs) and in the ultrafiltrate (Cu). The average percentage of added MPA recovered in the ultrafiltrate was calculated as: Fa = 100 × Cu/Cs.

2.4. Instrumentation

Separation of analytes was performed using a Kromasil C_8 (Eka Chemicals, Bohus, Sweden) column

(150 mm × 4.6 mm, 5 μm) preceded by a 0.5 μm precolumn filter (Waters, MA, USA). The HPLC system (Shimadzu, Japan) consisted of a LC-10AD pump, SIL-10A autoinjector and RF-10AXL fluorescence detector. Data were collected and analysed by Class LC-10 software (Version 1.63, Shimadzu, Kyoto, Japan).

The HPLC system was equilibrated with the mobile phase consisting of acetonitrile-32 mM glycine buffer, pH 9.2(20:80, v/v), at a flow rate of 1.0 ml/min.

The aqueous buffer solution (pH 9.2) consisted of 1000 ml of 40 mM glycine solution and 240 ml of 40 mM sodium hydroxide. The injection volume was 20 μl and the chromatographic peaks were detected at an excitation wavelength of 342 nm and an emission wavelength of 425 nm. The column temperature was maintained at 30 °C. For the determination of free MPA concentration, the sensitivity, a parameter of fluorescence detector, was set high, while the value was set medium for total MPA based on peak area as a measure of detector response. In addition, the gain, another parameter, was set at 1 in both situations.

2.5. Validation

The linearity of total and free MPA assay were assessed by analyzing calibration standards (1/*C* weighted) of MPA over the concentration range 0.05–40 mg/l and 5–1000 μg/l, respectively. The intra-day accuracy and precision were determined by assaying six replicates of QC samples in a single run. The inter-day accuracy and precision were evaluated by analyzing same QC samples and the procedure was repeated on different days ($n = 6$). Precision was characterized by the coefficients of variation (CV, %) whereas accuracy was expressed as a percentage error (PE, %) of nominal versus measured concentration.

2.6. Application

The HPLC method was applied to determination of the total MPA concentrations of patients treated with MMF from routine MPA monitoring. Meanwhile, the pharmacokinetics of MPA was investigated in five renal allograft recipients who received oral dosing of MMF (CellCept®, 750 mg, twice daily). Blood samples were collected into heparinized tubes on day 7 post-transplant, at times pre-dose (0) and 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0, and 12.0 h after administration. Plasma was immediately separated by centrifugation at 3000 × *g* for 10 min at 4 °C. Protein-free ultrafiltrate for each were prepared from 500 μl plasma using ultrafiltration through centrifree cartridges consisting 10,000 Da MW cutoff membrane (10,000 × *g*, 40 min at 25 °C). Plasma samples were stored at −20 °C until analysis. The Huashan hospital Ethnic Committee approved the design of the study and informed consent was obtained from the patients.

3. Results and discussion

3.1. Chromatography condition

By increasing the pH of mobile phase, the phenolic function of MPA became deprotonated because of solvatochromic effect. As a result, the retention of MPA decreased further and the molecule became highly fluorescent [26,27]. The ratio of the fluorescence yield at pH 7 and 9.2 was approximately 1:7, while the retention time decreased from 13.7 min at pH 7 to 3.1 min at pH 9.2. The use of low concentration of glycine buffer (pH 9.2, 32 mM) could achieve sufficient sensitivity to measure the MPA concentration in plasma. The mobile phase pH for Kromasil C_8 column is recommended between 2 and 9.5 to ensure maximum column life according to the fact sheet from manufacturer [28]. Furthermore, more than 1300 samples were injected onto the same column without loss in resolution. In conclusion, the Kromasil C_8 column was proved to be optimal because of chemical stability at this base condition (pH 9.2), separation from endogenous compounds and satisfactory run time without the addition of tetrabutylammonium bromide, an ion-pair reagent, reported by Hosotsubo [18].

Compared to the previous reported methods, which based on liquid–liquid extraction [11], solid-phase extraction [12] and protein precipitation with/without evaporation to dryness [15–17] for sample preparation of total MPA, the major advantage of our assay is the simpler, faster and less costly one-step extraction procedure in combination with a higher sensitivity and shorter run time. Typical chromatograms obtained from blank plasma, drug-free plasma spiked MPA and patient's plasma samples were presented in Figs. 1 and 2 . MPA and naproxen appeared as a well-separated peak with a retention time of 3.11 and 4.89 min. The run time for free and total MPA was 4 and 6 min, respectively. Plasma from ten transplant patients receiving other medications showed no interfering peaks were found at the retention time of MPA and I.S. Moreover, potentially coadministered drugs, such as cyclosporine A, tacrolimus, sirolimus, prednisolone, amlodipine, were not detected with the described analytical method.

3.2. Total MPA validation

The method reported here using constant volume (20 μl) directly to inject onto the column following simple protein precipitation with methanol was satisfactory. We also found naproxen as an internal standard with an appropriate retention time and suitable fluorescence properties. Based on direct comparison of peak areas after sample preparation versus the stock solution used to spiked the samples, the mean absolute recoveries of MPA were 97.26 ± 4.1%, 94.48 ± 1.4% and 98.27 ± 3.2% at concentrations of 0.1, 15 and 30 mg/l, respectively ($n = 6$). The mean absolute recovery of internal standard was found to be 98.31 ± 3.42% ($n = 10$). Furthermore, the limit of detection (LOD) was 8 μg/l (signal-to-noise ratio, 3), which was more sensitive than the results reported

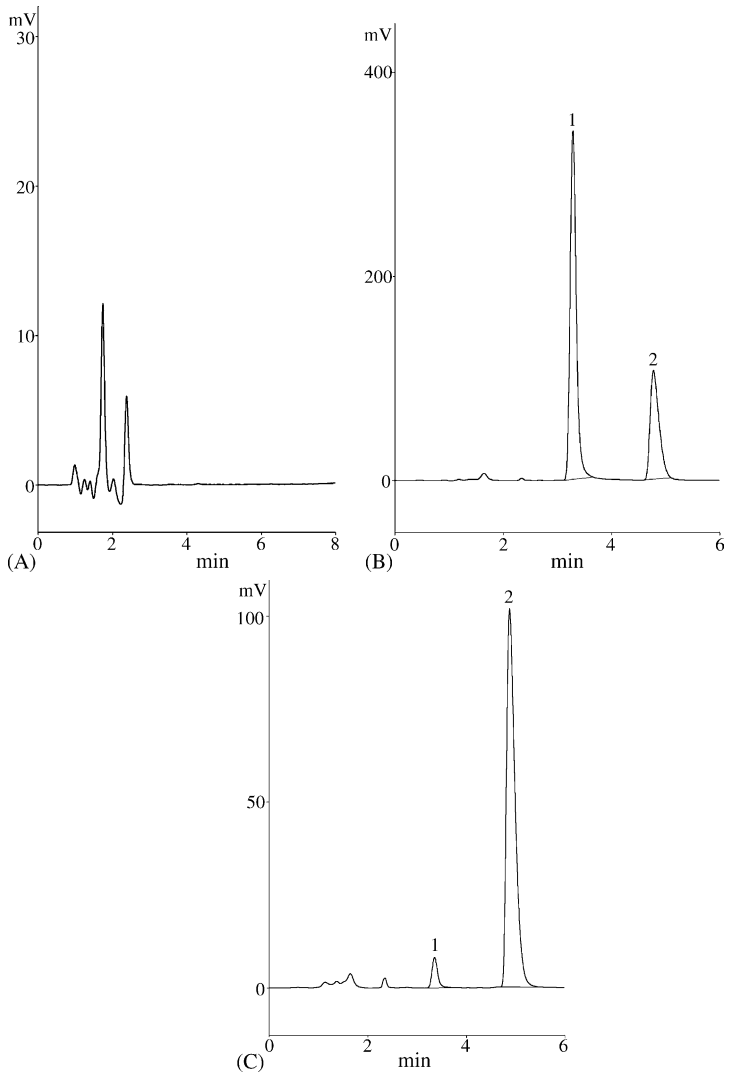

Fig. 1. Representative chromatograms for total MPA of (A) a blank plasma sample, (B) the same sample spiked with 25 mg/l of MPA and I.S. and (C) a patient plasma containing 0.68 mg/l of MPA 12 h after oral administration of MPA (750 mg, twice daily). Retention times of MPA (1) and internal standard (2) were 3.11 and 4.89 min, respectively.

in literatures coupled with UV detection [9–16] and fluorescence detection [18]. Although liquid chromatography with UV detection at 215 nm achieved a LOD of 10 μg/l, the shortcomings of this method involved multiple extraction steps in the sample pre-treatment and a relatively long run time of approximately 21 min [17].

There was a good linear relationship (1/C weighted) between peak area ratio(y) of total MPA to I.S. and $C(x)$ over the range of 0.05–40 mg/l. The mean correlation coefficients of calibration curves were more than 0.999. A typical calibration curve had the regression equation of $y = 0.1307x - 2.848 \times 10^{-3}$ ($r = 0.9999$).

The results of intra-day and inter-day accuracy and precision evaluation for QC samples were shown in Table 1. For MPA, both intra-day and inter-day precision CV% were all less than 7.0%, while those of accuracy PE% were less than 11%.

The samples whose concentrations were above the upper limit of the standard curve were estimated by determination of MPA in spiked plasma of 100 mg/l, which was diluted to 25 mg/l in blank plasma. The mean recovery of diluted samples was 95.92 ± 0.58% ($n = 6$).

3.3. Free MPA validation

The average percentage of added MPA recovered in the ultrafiltrate was 94.3 ± 3.6% at 0.1 mg/l, 97.6 ± 6.2% at 16 mg/l and 98.2 ± 2.2% at 32 mg/l. This indicated that the binding of MPA to the ultrafiltration system was insignificant.

A standard curve of directly injected ultrafiltrate (20 μl) was constructed by plotting peak areas versus concentrations of MPA over the range of 5–1000 μg/l. The relationship (1/C weighted) between the peak areas and

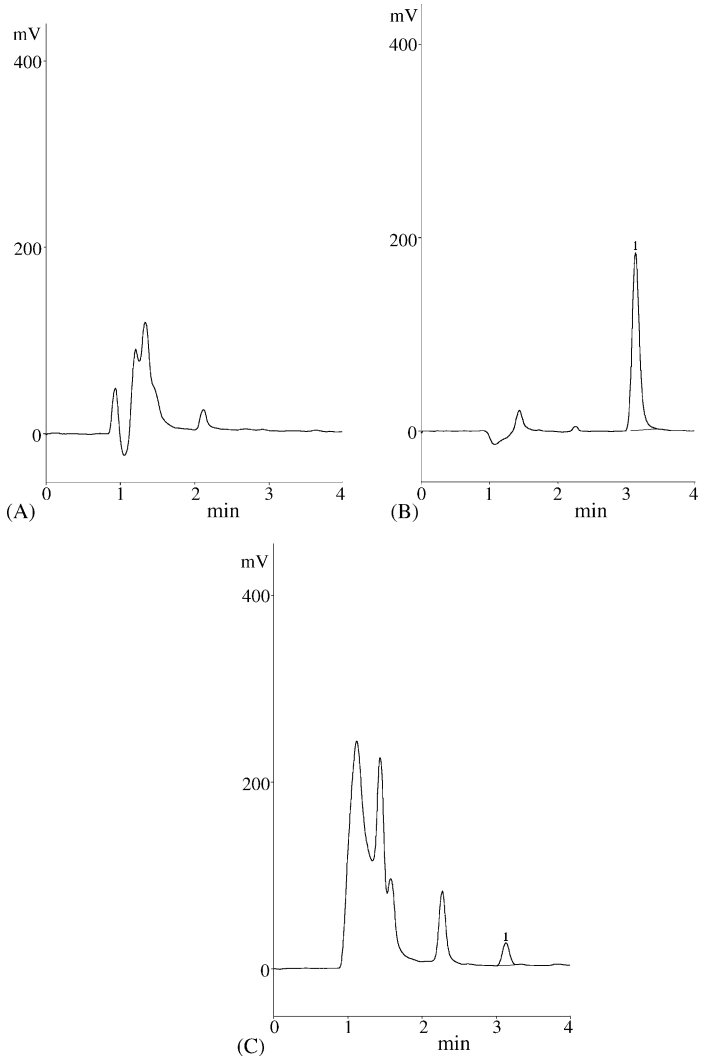

Fig. 2. Representative chromatograms for free MPA of (a) a ultrafiltrate sample from blank plasma, (b) the standard (100 μg/l) in sodium chloride solution (c) a patient plasma containing 23.09 μg/l of MPA 12 h after oral administration of MPA (750 mg, twice daily). Retention time of MPA (1) was 3.11 min.

concentrations was linear ($r > 0.99$). Although no internal standard was used here, there was an excellent correlation between the peak areas (y) and concentrations (x) as $y = 7568.5x - 1053.5$ ($r = 0.9991$). The limit of quantification (LOQ) was 5 μg/l, which compares favourably with the HPLC-UV method (LOD: 5 μg/l) of Shipkova et al. [17] and LC–MS methods (0.5–2.5 μg/l) [19–21]. Furthermore, the sample preparation is simpler and a lower LOQ of the present assay may be possible by increasing the volume of injection.

Table 1
Intra- and inter-assay precision and accuracy results of the total MPA

Theoretical value (mg/l)	Measured value (mean ± S.D.) (mg/l)	Precision CV (%)	Accuracy PE (%)
Intra-assay ($n = 6$)			
0.05	0.054 ± 0.002	3.48	8.0
0.1	0.102 ± 0.066	6.60	1.5
15	14.67 ± 0.27	3.14	−2.2
30	32.01 ± 0.48	1.51	6.7
Inter-assay ($n = 6$)			
0.05	0.055 ± 0.002	4.16	10.9
0.1	0.998 ± 0.011	1.12	−0.2
15	14.76 ± 0.71	4.82	−1.6
30	31.71 ± 1.13	3.56	5.7

CV: coefficient of variation; PE: percentage error.

Although the internal standard approach was suitable for the determination of free MPA concentration (data not shown), the method of directly injected ultrafiltrate was proved to be acceptable for assaying free MPA in plasma. Furthermore, the analysis was completed in 4 min and the sample pretreatment was simple. The results of intra-day and inter-day accuracy and precision evaluation for QC samples were shown in Table 2 . For free MPA, both intra-day and inter-day precision CV% were all less than 10%, while those of accuracy PE% were less than 10%. Due to the ease of the procedure, the method described here appeared suitable for large-scare analyses and therapeutic drug monitoring.

Table 2
Intra- and inter-assay precision and accuracy results of the free MPA

Theoretical value (μg/l)	Measured value (mean ± S.D.) (μg/l)	Precision CV (%)	Accuracy PE (%)
Intra-assay ($n = 6$)			
5	4.772 ± 0.237	4.98	−4.6
8	7.465 ± 0.475	6.37	−6.7
400	388.9 ± 5.616	1.44	−2.8
800	830.3 ± 17.70	2.03	3.8
Inter-assay ($n = 6$)			
5	4.911 ± 0.407	8.28	−1.8
8	7.571 ± 0.445	5.87	−5.4
400	367.8 ± 23.08	6.28	−8.0
800	823.1 ± 54.76	6.65	2.9

CV: coefficient of variation; PE: percentage error.

3.4. Stability

The stability of stock solutions of MPA in methanol was checked and proved to be stable for at least 6 months at 4 °C. The analytes reconstituted in the mobile phase were also stable at ambient conditions (no control of temperature in the autosampler) for at least 24 h, thus allowing us to automate the procedure. No significant degradation in plasma samples (spiked or clinical) during 6 months at −20 °C was detected. Furthermore, MPA was stable for at least three freeze–thaw cycles.

3.5. Application

The method has been applied successfully to determine the total MPA concentrations in 756 samples, which were drawn at pre-dose and 2 h post-dose. The total MPA concentrations range from 0.06 to 34.25 mg/l (median 2.92 mg/l). On the other hand, a 12 h pharmacokinetic profile of total and free MPA was investigated in five transplant patients receiving a triple immunosuppressive therapy with MMF, prednisolone, and cyclosporine A. The concentration (mean ± S.E.)–time (h) profile of MPA was illustrated in Fig. 3. The results indicated that the total MPA concentrations ranged from 0.20 to 15.47 mg/l and free drug concentrations ranged from 6.16 to 967.5 μg/l. In these samples, the average percentage of free MPA was 3.28 ± 1.21%.

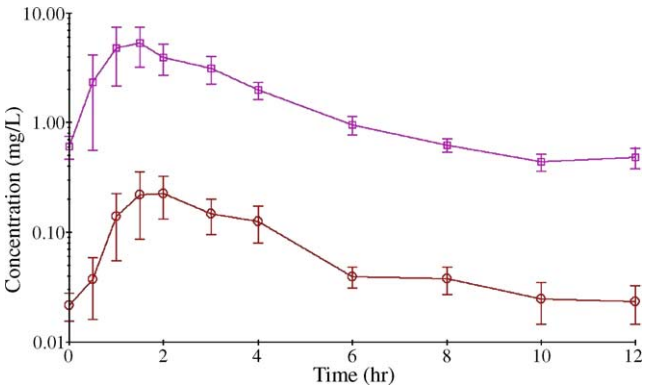

Fig. 3. Twelve-hour pharmacokinetic profiles of MPA from five renal transplant recipients receiving chronic oral dosing of MMF (CellCept®, 750 mg twice daily) co-administered with cyclosporine and prednisolone (◯, free MPA concentration; □, total MPA concentration).

4. Conclusion

Simultaneously measuring total and free concentrations of MPA should provide a more complete understanding of the MPA pharmacokinetic variability observed in transplant populations. The present method, to our knowledge, is the first analytical method described for the quantification of free MPA with fluorescence detection. Due to its high sensitivity, the assay may also provide a useful tool for drug analysis in small volume of plasma. Other advantages include the simplicity of preparation, the shorter run time, and the good reproducibility. Furthermore, this HPLC method is reliable and ideal for large-scale analyses and routine monitoring of free and total MPA in patients' plasma.

Acknowledgements

This study was supported by Shanghai Natural Science Fund Grant (number 032R14010). We thank Dr. Fu-ming Lu and Jian-yong Zhong for their clinical investigations. We also gratefully acknowledge the expert technical assistance of Yan Zhong, Dan-yi Chi, Zhi-wei Gao, Zhong-dong Li, and Xiao-jin Shi.

References

[1] R.E. Bullingham, A.J. Nicholls, B.R. Kamm, Clin. Pharmacokinet. 34 (1998) 429.
[2] A.G. Johnson, R.J. Rigby, P.J. Taylor, C.E. Jones, J. Allen, K. Franzen, M.C. Falk, D. Nicol, Clin. Pharmacol. Ther. 66 (1999) 492.
[3] L.M. Shaw, A. Nawrocki, M. Korecka, S. Solari, J. Kang, Ther. Drug Monit. 26 (2004) 347.
[4] L.M. Shaw, T. Pawinski, M. Korecka, A. Nawrocki, Ther. Drug Monit. 24 (2002) 68.
[5] 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.
[6] D.W. Mudge, B.A. Atcheson, P.J. Taylor, P.I. Pillans, D.W. Johnson, Ther. Drug Monit. 26 (2004) 453.
[7] B.A. Atcheson, P.J. Taylor, C.M. Kirkpatrick, S.B. Duffull, D.W. Mudge, P.I. Pillans, D.W. Johnson, S.E. Tett, Ther. Drug Monit. 26 (2004) 284.
[8] B. Kaplan, H.U. Meier-Kriesche, G. Friedman, S. Mulgaonkar, S. Gruber, M. Korecha, K.L. Brayman, L.M. Shaw, J. Clin. Pharmacol. 39 (1999) 715.
[9] D. Teshima, N. Kitagawa, K. Otsubo, K. Makino, Y. Itoh, R. Oishi, J. Chromatogr. B 780 (2002) 21.
[10] C.G. Zambonin, A. Aresta, F. Palmisano, J. Chromatogr. B 806 (2004) 89.
[11] K. Na-Bangchang, O. Supasyndh, T. Supaporn, V. Banmairuroi, J. Karbwang, J. Chromatogr. B 738 (2000) 169.

[12] D. Teshima, K. Otsubo, N. Kitagawa, S. Yoshimura, Y. Itoh, R. Oishi, J. Clin. Pharm. Ther. 28 (2003) 17.
[13] A. Pastore, A. Lo-Russo, F. Piemonte, L. Mannucci, G. Federici, J. Chromatogr. B 776 (2002) 251.
[14] J.F. Gummert, U. Christians, M. Barten, H. Silva, R.E. Morris, J. Chromatogr. B 721 (1999) 321.
[15] V. Srivatsan, A.K. Dasgupta, P. Kale, R. Verma, P. Joshi, D. Soni, M. Patel, G. Soni, J. Patel, H. Modi, J. Chromatogr. A 1031 (2004) 259.
[16] D.A. Saunders, J. Chromatogr. B 704 (1997) 379.
[17] M. Shipkova, P.D. Niedmann, V.W. Armstrong, E. Schutz, E. Wieland, L.M. Shaw, M. Oellerich, Clin. Chem. 44 (1998) 1481.
[18] H. Hosotsubo, S. Takahara, Y. Kokado, S. Permpongkosol, J.D. Wang, T. Tanaka, K. Matsumiya, M. Kitamura, A. Okuyama, H. Sugimoto, J. Pharm. Biomed. Anal. 24 (2001) 555.
[19] B. Atcheson, P.J. Taylor, D.W. Mudge, D.W. Johnson, P.I. Pillans, S.E. Tett, J. Chromatogr. B 799 (2004) 157.
[20] C. Willis, P.J. Taylor, P. Salm, S.E. Tett, P.I. Pillans, J. Chromatogr. B 748 (2000) 151.
[21] F. Streit, M. Shipkova, V.W. Armstrong, M. Oellerich, Clin. Chem. 50 (2004) 152.
[22] R. Mandla, P.D. Line, K. Midtvedt, S. Bergan, Ther. Drug Monit. 25 (2003) 407.
[23] J.L. Beal, C.E. Jones, P.J. Taylor, S.E. Tett, Ther. Drug Monit. 20 (1998) 685.
[24] L.T. Weber, M. Shipkova, V.W. Armstrong, N. Wagner, E. Schutz, O. Mehls, L.B. Zimmerhackl, M. Oellerich, B. Tonshoff, Clin. Chem. 48 (2002) 51.
[25] I. Nowak, L.M. Shaw, Clin. Chem. 41 (1995) 1011.
[26] U.D. Renner, C. Thiede, M. Bornhauser, G. Ehninger, H. Thiede, Anal. Chem. 73 (2001) 41.
[27] R.J. Bopp, R.E. Schirmer, D.B. Meyers, J. Pharm. Sci. 61 (1972) 1750.
[28] http://www.kromasil.com/products/techsupp/index.htm.