—Regular Articles—

Quantification and 24-hour Monitoring of Mycophenolic Acid by High-Performance Liquid Chromatography in Japanese Renal Transplant Recipients

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We developed a rapid, simple, and sensitive high-performance liquid chromatography method with UV detection for the quantitative determination of mycophenolic acid (MPA) in human plasma. MPA and the internal standard (naproxen) were separated using a mobile phase of 0.04 M H_3PO_4-acetonitrile-methanol (3 : 3 : 4 v/v/v) over a CAPCELL PAK C18 MG column. A flow-rate of 0.5 ml/min was used at ambient temperature and sample detection was carried out at 254 nm. The assay required only 100 μl of plasma and involved liquid-liquid extraction, which gave high recovery (>94%). The lower limit of quantification for MPA was 0.05 μg/ml. Inter- and intra-day coefficients of variation were less than 9.6% and accuracies were within 9.3%. Additionally, we validated this method in 24-hour monitoring of plasma MPA concentrations after mycophenolate mofetil (MMF) morning and evening administration in 40 Japanese renal transplant recipients with 1.5 g/day MMF. The time to reach the maximum (11.7 μg/ml) and second peak (4.5 μg/ml) of MPA after morning 0.75 g MMF administration was 2.6 h and 9.0 h, and time to reach maximum (10.5 μg/ml) after evening 0.75 g administration was 4.0 h.

Key words——mycophenolic acid; high performance liquid chromatography; 24h-monitoring

INTRODUCTION

Mycophenolic acid (MPA), the active metabolite of mycophenolate mofetil (MMF), is a cornerstone immunosuppressive drug effectively used in renal transplantation.[1,2] MPA is primarily metabolized by glucuronidation of the phenolic hydroxy group by uridine diphosphate-glucuronosyltransferases (UGTs) to an inactive mycophenolic acid glucuronide (MPAG), which is the major uninary excretion product of MPA.[3,4] UGT1A9 is identified as the main UGT isoforms involved in MPA glucuronidation.[5—7] MPA pharmacokinetics is characterized by a large inter- and intra-individual variability in plasma concentration, thereby underlining the need for therapeutic drug monitoring in renal recipients in order to individualize dosage.[8,9] Generally, patients with low area under plasma concentration-time curves (AUC) of MPA have an increased risk of graft rejection, whereas a high AUC of MPA is associated with increased risk of toxicity.[10—12] Kuypers et al. have reported that the *−275 T/A* and *−2152 C/T* SNPs of the *UGT1A9* gene promoter are associated with significantly lower MPA concentration in white renal recipients after oral administration of MMF.[13] Therefore the individual difference of plasma MPA concentration seems to be caused by UGT genetic polymorphism.

On the other hand, several high-performance liquid chromatography (HPLC) methods for the quantitation of MPA and its glucuronide metabolites in plasma have been reported.[14] Some methods allow quantitation only of MPA concentration,[15—18] and several permit the simultaneous determination of MPA and MPAG.[19—26] Most of these methods are time-consuming extraction step and require larger sample volumes, special techniques and complicated procedures for the assay. So a simpler assay method is highly expected. The method presented here is rapid and simple, and consists of a liquid-liquid extraction followed by HPLC-UV, which allows the determination of MPA concentration in human plasma. The extraction procedure used for the pre-treatment plasma sample ensures high recovery from a relatively small amount of plasma (100 μl) for complete analysis. We validated this HPLC-UV method by investigating 24-hour MPA pharmacokinetics in human renal transplant recipients. In addition, we show circadian

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rhythms of MPA in the renal transplant recipients using this assay.

EXPERIMENTAL

Reagents and Chemicals MPA (6-(4-hydroxy-6-methoxy-7-methyl-3-oxo-5-phtalanyl)-4-methyl-4-hexenoic acid) was donated by Roche Pharmaceutical (Palo Alto, CA). All other reagents and chemicals were purchased from Nacalai Tesque (Kyoto, Japan). All solvents were of HPLC grade.

HPLC System A PU-2080 plus chromatography pump (JASCO) equipped with a UV-2075 plus ultraviolet detector (JASCO) was used. The HPLC column used was a CAPCELL PAK C18 MG (250 mm×3.0 mm I.D., Shiseido, Tokyo, Japan) with a mobile phase consisting of 0.04 M H_3PO_4-acetonitrile-methanol (3 : 3 : 4, v/v/v), which was degassed in an ultrasonic bath prior to use. A flow-rate of 0.5 ml/min was used at ambient temperature and sample detection was carried out at 254 nm.

Extraction Method Following the addition of naproxen (1 μg) in methanol (10 μl) to 100 μl plasma samples as an internal standard, 1.0 ml acetonitrile was added to the plasma samples and the solution was vortexed for 30 s. This mixture was centrifuged for 5 min at 13,000×g. Aliquots of 15 μl of the clear supernatant filtered through Millipore filters (0.45 μm, Millex-LH®, Japan) were then directly injected into the HPLC apparatus.

Calibration Graph Stock solution for generating standard curve of MPA was prepared by dissolving the dry reagents in methanol to yield concentrations of 1.0 mg/ml. Working standard solutions of MPA (0.05, 0.5, 1.0, 5.0, 10 and 50 μg/ml) were prepared by serial dilution with methanol. Stock solutions are stable at 4°C for at least 3 months according to the FDA.[27] A calibration curve was obtained for spiked blank plasma samples in a concentration range of 0.05—50 μg/ml for MPA. Blank plasma samples were treated as described above. A calibration graph was constructed from the peak-height ratio of MPA to the naproxen internal standard from the HPLC chromatograms and then plotted against the nominal MPA concentration.

Recovery Recovery following the extraction procedure was determined by comparing the peak areas of blank plasma samples extracted according to the above procedure with those of non-extracted control samples.

Assay Validation Inter-day precision and accuracy were evaluated from the analysis of control samples measured on five different days, whereas intra-day precision and accuracy were evaluated by analyzing spiked controls five times over the course of one day in random order. The precision of the HPLC method at each concentration was evaluated by comparing the coefficient of variation (CV) (obtained by calculating the standard deviation (SD) as a percentage of the mean calculated concentration) with the accuracy estimated for each spiked control (obtained by comparing the nominal concentration with the assayed concentration). Limits of quantification (LOQ) were determined as the lowest non-zero concentration measured with an intra-day C.V. of $<20\%$ and an accuracy of $<\pm 20\%$,[28] and limits of detection (LOD) were determined as the lowest concentration with a signal to noise ratio of three.

Application to Pharmacokinetics Study The HPLC method was used to quantitate the plasma concentrations of MPA in renal transplant recipients. This study was approved by the Ethics Committee of Akita University Hospital, and all patients gave written informed consent. Renal transplant recipients were given combination immunosuppressive therapy consisting of tacrolimus and 1.5 g/day of MMF (Cellcept®) as equally divided doses every 12 hours at a designated time (09 : 00 and 21 : 00). The daily tacrolimus dose was adjusted according to the clinical state of the recipient, with the whole blood through target level being 10—15 ng/ml. In addition, all recipients were concomitantly given 10 mg/day of prednisolone. Meals were served at 7 : 30, 12 : 30, and 18 : 00 daily. While meal content (Japanese food) varied each day for each patient, energy, fat, protein, and water contents were standardized (energy: 1700—2400 kcal, protein: 70—90 g, fat: 40—50 g, water: 1600—2000 ml) according to body weight. On day 28 after renal transplantation, whole blood samples (5 ml) were collected by vein puncture just prior to and at 1, 2, 3, 4, 6, 9 and 12 hours after oral MMF administration at 9 : 00 and 21 : 00. Plasma was isolated by centrifugation at 1,900×g for 15 min and stored at −30°C until analysis. Patient plasma samples (100 μl) were then extracted as described above in Extraction Method and injected into the HPLC system. Pharmacokinetic analysis of MPA was carried out according to a standard non-compartmental method using WinNonlin software (Phar-

sight Co., CA, version 4.0.1). Values for the maximum plasma concentration (C_{max}) and time to reach the maximum (t_{max}) were obtained directly from the profile.

RESULTS AND DISCUSSION

Chromatograms We describe a simple and sensitive HPLC-UV method for the determination of MPA concentrations in human plasma. Our HPLC assay regulated detectability by adding 1 ml of acetonitrile as extract. The assay was able to quantitate MPA in a plasma volume as low as 10 μl by adding 100 μl of acetonitrile as extract; however, we optimized the assay for 100 μl plasma to reduce a measurement error. Typical chromatograms obtained for blank plasma and for plasma samples spiked with MPA (1 μg/ml) are shown in Figs. 1(A) and 1(B), respectively. Retention times of MPA and internal standard were 5.4 and 6.4 min, respectively. The presented method requires only 100 μl sample volume, takes 10 min for preparation of a batch of 15 samples/one patient and 10 min for a single chromatographic development. This assay is extremely speedier than the previous methods.

Calibration Curve The calibration curve for MPA in plasma was found to be linear over the concentration range of 0.05—50 μg/ml. The typical calibration curve (obtained using the least-squares method) for MPA could be expressed by the equation: $Y=0.1858X+0.0062$ ($\gamma^2=0.9999$), with Y being the peak height ratio and X being the concentration in μg/ml.

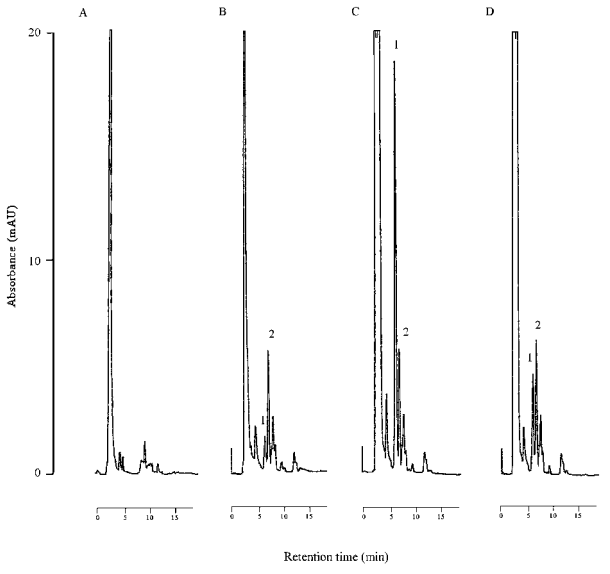

Fig. 1. Representative Chromatograms of A) Plasma Blank, B) 100 μl Plasma Spiked with MPA (0.1 μg) and Naproxen (1 μg), C) Plasma Sample at 2 h (Calculated Concentrations of MPA: 17.8 μg/ml) and D) Plasma Sample at 12 h (Calculated Concentrations of MPA: 3.7 μg/ml) after Oral 0.75 g MMF Administration to Patient
Peaks: 1: MPA, 2: naproxen (IS).

Recovery The recovery of MPA from human plasma was determined by adding five known MPA concentrations (0.5, 1, 5, 10 and 50 μg/ml) to drug-free plasma samples. Mean extraction recovery values for MPA were 94—103%, within a concentration range of 0.5—50 μg/ml (Table 1). This method was extremely simple preparation procedure using only acetonitrile as extract and had high recovery for MPA.

Precision and Accuracy The coefficients of variation (CV) and accuracy for intra- and inter-day assays were determined at concentrations of 0.5—50 μg/ml for MPA. CV values and accuracies for intra- and inter-day assays were less than 9.6% and 9.3%, respectively (Table 1). The precision and accuracy of this HPLC assay is suitable for both routine therapeutic drug monitoring applications and clinical pharmacokinetic studies.

Sensitivity Values for the lower limit of quantification (LOQ) and limit of detection (LOD) for MPA from 100 μl of plasma were 0.05 μg/ml and 0.01 μg/ml, respectively. The limit of quantification of 0.05 μg/ml for MPA in this HPLC-UV method was more sensitive than that of previous methods.[16—18,21,22,25]

Application Chromatograms of plasma samples collected from a representative patient at 2 and 12 hours after oral administration of MMF are shown in Figs. 1(C) and 1(D), respectively. The patients received a triple immunosuppressive therapy with MMF, tacrolimus and prednisolone, and no interference between MPA and the biological matrix was observed (Figs. 1(C) and 1(D)). Futhermore, renal transplant recipients were receiving tacrolimus and prednisolone for immunosuppression. In present method, however, no analytical interference with these compounds was found (Figs. 1(C) and 1(D)).

Figure 2 showed time course of the mean plasma MPA concentrations in Japanese 40 renal transplant recipients. The MPA concentration in individual plasma samples ranged from 0.7 to 36.5 μg/ml. The assay sensitivity of less than 0.5 μg/ml was sufficient for MPA monitoring in renal transplant recipients. Our

Table 1. Accuracy and Precision of HPLC Assay for the Determination of MMF in Human Plasma ($n=5$)

Added μg/ml	Intra-day			Inter-day			Recovery (%)
	Found mean±SD	CV (%)	Accuracy (%)	Found mean±SD	CV (%)	Accuracy (%)	
0.50	0.48±0.04	8.3	−4.1	0.51±0.04	7.8	2.1	97
1.00	1.07±0.11	9.6	6.9	0.97±0.09	9.3	−2.9	98
5.00	5.13±0.48	9.4	2.6	5.18±0.38	7.3	3.6	103
10.0	9.25±0.54	5.8	−7.5	9.86±0.74	7.5	−1.4	94
50.0	49.5±4.3	8.7	−1.0	47.8±3.8	7.9	−4.4	96

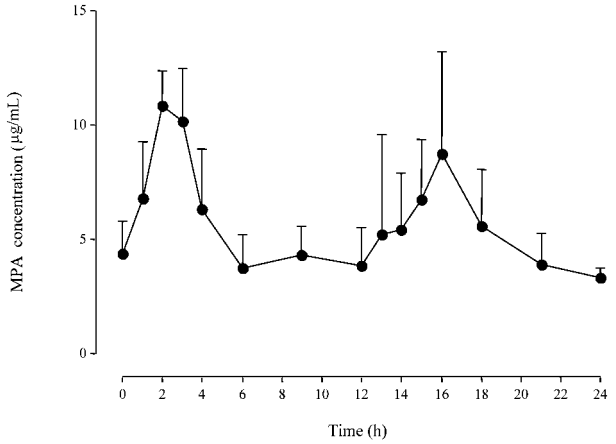

Fig. 2. Mean (±SD) Plasma Concentration-time Profiles of MPA during Repetitive Oral MMF Administration as Equally Divided Doses (0.75 g) Every 12 Hours at a Designated Time (09 : 00 and 21 : 00) to Japanese Renal Transplant Recipients ($n=40$)

Table 2. Pharmacokinetic Parameters of MPA in Renal Transplant Recipients

Study group	Daytime	Nighttime
Dose (mg/kg)	14.4±2.5	14.4±2.5
AUC_{0-12} (μg·h/ml)	71.1±25.0	69.4±24.3
C_{max} (μg/ml)	11.7±1.4	10.5±4.2
C_0 (Trough) (μg/ml)	3.2±1.3	2.8±0.6
t_{max} (h)	2.6±0.5	4.0±1.8
CL/F (L/h)	11.7±4.1	12.1±4.3

The values are shown as the mean±S.D. C_{max}: maximum plasma concentration, AUC_{0-12}: area under the plasma concentration-time curve from 0 to12 h, CL/F: total body clearance.

HPLC method was applied successfully to determine the 24-hour pharmacokinetic profile of MPA in renal transplant recipients (LOQ: 0.05 μg/ml). The present method is suitable for transplant patients receiving a triple immunosuppressive therapy with MMF, tacrolimus and prednisolone and sensitive enough for monitoring MPA.

The MPA pharmacokinetic parameters are given in Table 2. The mean AUC_{0-12} values after the morning and night doses were 71.1 and 69.4 μg·h/ml, respectively. The mean maximum MPA concentrations (C_{max}) after the morning and night doses were 11.7 and 10.5 μg/ml, respectively. The oral clearances (CL/F) of MPA in the daytime and nighttime were 11.7 and 12.1 l/h, respectively. To our knowledge, there is little pharmacokinetic study of MPA in renal transplant recipients treated with of low-dose MMF (1.5 g/day). This method is already being used to measure plasma MPA concentrations in renal transplant recipients. The results of these investigations will be reported in a separate paper.

In contrast, t_{max} of MPA was longer after evening administration than morning because of delayed absorption (4.0 vs. 2.6 h, $p=0.0377$). The reduction of absorption and delay of absorption may result from the reduced gastric emptying rate due to physiologically slower enterokinesis in the nighttime than in the daytime. Furthermore, the second peak of MPA concentration was observed at 9 h after morning MMF administration, but not after evening administration. MPA is known to undergo an enterohepatic recirculation.[3,29] An enterohepatic recirculation of MPA might have higher activity in the daytime than the nighttime.

CONCLUSION

Our HPLC method was applied to the pharmacokinetic study of MPA in renal transplant recipients that also received tacrolimus for immunosuppression and prednisolone, and no interference between MPA and the biological matrix was observed. MMF is generally administered as equally divided doses of drug twice daily. However, pharmacokinetic monitoring has not been fully examined for both morning and evening administrations. The HPLC method described here

was used to measure 24-hour MPA plasma concentrations in renal transplant recipients. The present new HPLC-UV method is simple and rapid considering the sample treatment procedure. The method is suitable for MPA monitoring in renal transplant recipients and sensitive enough for MPA monitoring during pharmacokinetics studies.

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