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Determination of a new immunosuppressant, mycophenolate mofetil, and its active metabolite, mycophenolic acid, in rat and human body fluids by high-performance liquid chromatography

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Abstract

Mycophenolate mofetil, a new immunosuppressant, is a morpholinoethyl ester of mycophenolic acid. A new selective, sensitive and simple high-performance liquid chromatographic method was developed for the determination of mycophenolic acid and mycophenolate mofetil in biological samples. The preparation of samples was based on liquid–liquid extraction. The compounds were separated on a CN column using acetonitrile–0.01 *M* phosphate buffer (1:4, v/v) as the mobile phase. UV detection was used at wavelengths 215 and 304 nm. The detection limit was 5 ng per injection volume. This method enabled pharmacokinetic and pharmacodynamic studies in humans and rats.

1. Introduction

Mycophenolate mofetil (MPM, Fig. 1a), a new immunosuppressant, is a morpholinoethyl ester of mycophenolic acid (MPA, Fig. 1b), which is its active metabolite. MPA, a fermentation product of several *Penicillium* species, is a potent, noncompetitive, reversible inhibitor of eukaryotic inosine monophosphate dehydrogenases. MPA thus inhibits the synthesis of guanosine monophosphate. This enzyme plays an important role in the purine metabolism of lymphocytes [1,2]. Clinical studies of MPM in human organ transplant recipients are now in progress [3,4].

Several methods have already been used for such determinations. In an *in vitro* study of MPA as an antibiotic, thin-layer chromatography (TLC) was used by Noto *et al.* [5]. A gas chromatographic (GC) method for MPA [6,7] has been used for human plasma and a high-performance liquid chromatographic (HPLC) method for fermentation broth [8]. For biological samples obtained after oral administration of

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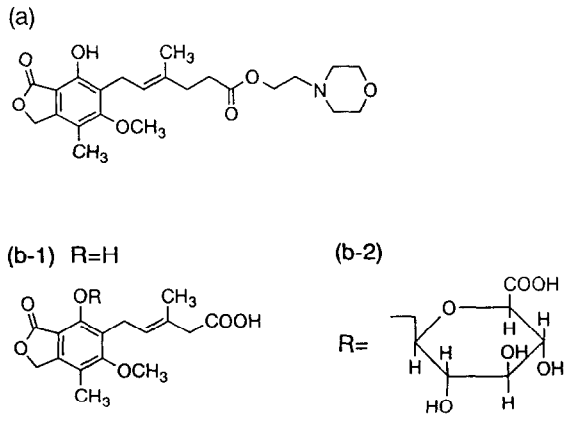

Fig. 1. Structures of MPM (a), MPA (b1), and MPA glucuronide (b2).

MPM the use of an HPLC assay is only reported in one study [9]. However, the sensitivities of these methods are low. The performance of both pharmacokinetic and pharmacodynamic studies in humans and in experimental animals such as rats, requires new highly sensitive methods that are suitable for many clinical and laboratory samples. In studies of small animals, it is difficult to start an assay from one ml of biological matrix. Therefore, a highly sensitive assay in which it is possible to determine MPA from a small sample volume of, for example, 0.1 ml of rat plasma, is required. For such studies in humans, the fact that transplant patients do not receive only one immunosuppressant, but may receive several–such as steroids, cyclosporin A and azathioprine–must be taken into account. Interfering peaks caused by immunosuppressants administered concomitantly with MPM should not be detected on the chromatograms in the assay.

This report describes a selective and sensitive isocratic HPLC assay for the determination of both MPM and MPA in rat plasma, bile and tissue homogenate and in human plasma. Furthermore, the determination of the major metabolite of MPA, a phenolic glucuronide (Fig. 1b), has been challenged by enzymatically cleaving the glucuronide.

2. Experimental

2.1. Chemicals and reagents

MPM was kindly supplied by Syntex Research (Palo Alto, CA, USA). MPA was purchased from Sigma (St. Louis, MO, USA). Diazepam and indomethacin were used as internal standards. Diazepam was purchased from Wako Pure Chemicals (Osaka, Japan). β-Glucuronidase (from *Helix pomatia*, crude solution, 100 000 units/ml), indomethacin, acetonitrile (HPLC-grade), and water (HPLC-grade) were obtained from Nacalai Tesque (Kyoto, Japan). All other reagents were of reagent grade.

2.2. HPLC equipment

The HPLC system (Shimadzu, Kyoto, Japan) consisted of an LC-10A liquid delivery module, an SPD-10A ultraviolet detector, a CTO-10A column oven and a Shim-pack CLC-CN column (150 × 6.0 mm I.D., 5 μm particle size). Samples were injected with a SIL-10A automatic injector. The system was controlled with an SCL-10A system controller under the following conditions: flow-rate, 1.0 ml/min; column temperature, 50°C; detection wavelength, 215 nm for rat plasma and 304 nm for rat bile, rat tissue homogenate and human plasma; injection volume, 30 μl. The area under each peak was calibrated with a Shimadzu data processor, CR-5A Chromatopack.

2.3. Mobile phase and reagent solution

The mobile phase was 0.01 *M* phosphate buffer pH 5.0–acetonitrile (4:1, v/v). This mixture was filtered and degassed by vacuum and sonication. The internal standards, diazepam and indomethacin, were dissolved in methanol and stored at 4°C until used. Stock solutions of MPM and MPA in methanol were stored in amber bottles at 4°C and used to prepare calibration standards.

The calibration curve samples were prepared by adding known amounts of MPM and MPA to

the body fluids or tissue homogenate. These samples were then treated by the extraction procedure described below. β-Glucuronidase solution for the enzymatic treatment of rat and human plasma was prepared by dissolving the enzyme in 0.1 *M* phosphate buffer (pH 6.0) to a concentration of 105.2 units/ml. This solution was used immediately after preparation.

2.4. Preparation of rat tissue homogenate

Male Wistar rats weighing 300–350 g were used. The animals were killed by cervical dislocation, and their livers and kidneys were then perfused with ice-cold 0.05 *M* Tris-HCl buffer (pH 7.4) containing 1.15% KCl, and homogenized with a glass homogenizer in the same ice-cold solution (1:4, w/v). Epithelial cells from the small intestine obtained by using a cover glass, were directly homogenized in the same ice-cold solution (1:4, w/v) with a glass homogenizer. Homogenates were obtained after centrifuging at 1000 *g* for 5 min. The reason we used ice-cold buffer was to avoid progression of the enzyme reaction before extraction.

2.5. Extraction of samples of body fluids and tissue homogenates

Rat plasma

To 50 μl of rat plasma in a conical centrifuge tube (1.5 ml) was added 200 μl of methanol and 50 μl of diazepam solution (6 μg/ml) as an internal standard, these components were mixed well. After centrifugation at 3000 *g* for 10 min, 250 μl of the supernatant was transferred into a clean conical glass centrifuge tube (10 ml), to which 2.5 ml of chloroform and 1 ml of 0.01 *M* phosphate buffer (pH 3.0) were added, the contents of the tube were mixed well with a reciprocating shaker for 30 min. After centrifugation at 3000 *g* for 10 min, 2 ml of the organic phase was transferred to a clean tube and evaporated to dryness under a stream of nitrogen gas. The residue was redissolved in 50 μl of acetonitrile. A 30-μl volume of the resulting solution was injected onto the HPLC system.

Rat bile

To 50 μl of rat bile in a conical centrifuge tube (1.5 ml) was added 200 μl of methanol and 50 μl of indomethacin solution (100 μg/ml) as an internal standard, these components were mixed well. After centrifugation at 3000 *g* for 10 min, 250 μl of the supernatant was transferred to a clean conical glass centrifuge tube (10 ml). A 3-ml volume of 0.01 *M* phosphate buffer (pH 9.0) and 3 ml of diethyl ether were then added and the contents of the tube were mixed well for 10 min and centrifuged at 3000 *g* for 10 min, after which the organic phase was aspirated. Subsequently, 3 ml of *n*-heptane was added, and the contents of the tube were treated in the same way. The aqueous phase was adjusted to pH 3.0 with a drop of 1 *M* HCl, after which 3 ml of chloroform was added; the components were mixed well for 30 min, the resulting mixture then being centrifuged at 3000 *g* for 10 min. The aqueous phase was aspirated and 2 ml of the organic phase was transferred to a clean tube. After being evaporated to dryness, the residue was redissolved in 100 μl of acetonitrile, and 30 μl of the resulting solution were injected onto the HPLC system.

Rat tissue homogenates

To 0.5 ml of each homogenate (rat liver and kidney, or epithelial cells of rat small intestine) in a conical glass centrifuge tube (10 ml) was added 2 ml of methanol and 50 μl of diazepam solution (200 μg/ml) as an internal standard. These components were mixed well and centrifuged at 3000 *g* for 20 min; 2 ml of the supernatant were then transferred to a clean glass tube (20 ml). Five ml of chloroform and 10 ml of 0.01 *M* phosphate buffer (pH 3.0) were then added to the tube, and the contents were mixed well for 30 min. After centrifugation at 3000 *g* for 10 min, 4 ml of the organic phase were transferred to a clean tube and evaporated to dryness. The residue was redissolved in 100 μl of

acetonitrile, and 30 μl of the resulting solution were injected onto the HPLC system.

Human plasma

To 1 ml of human plasma in a conical glass centrifuge tube (10 ml) were added 2 ml of methanol and 50 μl of diazepam solution (50 μg/ml) as an internal standard. These components were mixed well and centrifuged at 3000 *g* for 20 min, and 2 ml of the supernatant were then transferred to a clean conical glass centrifuge tube (20 ml). Chloroform (5 ml) and 10 ml of 0.01 *M* phosphate buffer (pH 3.0) were then added and the tube contents were mixed well for 30 min and centrifuged at 3000 *g* for 10 min. A 4-ml volume of the organic phase was then transferred to a clean tube and evaporated to dryness under a stream of nitrogen gas. The residue was redissolved in 50 μl of acetonitrile, and 30 μl of the resulting solution were injected onto the HPLC system.

Recovery

The extraction efficiency was evaluated by spiking a known amount of MPA into rat plasma, bile, liver homogenate and human plasma. The samples were treated as described above, and the peak areas of MPA were compared with the peak areas obtained from the buffered sample to which the same amount of MPA was added and which was extracted with the same method. The absolute recovery was also evaluated by comparing with aqueous non-extracted standard.

2.6. Conditions for enzymatic hydrolysis of MPA glucuronide in rat and human plasma

We used β-glucuronidase for estimating the total plasma concentration of MPA. To 50 μl of rat bile, rat plasma or human plasma in a conical glass centrifuge tube (10 ml), we added 950 μl of β-glucuronidase solution. This mixture was incubated for 1 h at 37°C, and the resulting solution was then used for the extraction of MPA, as described above.

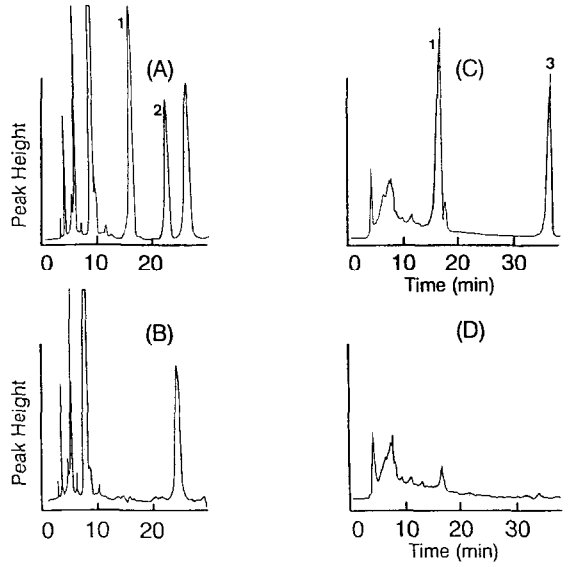

Fig. 2. Chromatograms of biological samples. (A) Rat plasma spiked with MPA (2.0 μg/ml); (B) blank rat plasma sample; (C) rat bile spiked with MPA (2.0–50.0 μg/ml); (D) blank rat bile sample. Peaks: 1 = MPA; 2 = diazepam (internal standard); 3 = indomethacin (internal standard).

3. Results and discussion

Fig. 2 shows typical chromatograms of the samples obtained from 50 μl of rat plasma (A) and from 50 μl of rat bile (C) spiked with MPA at concentrations of 2.0 μg/ml and 50.0 μg/ml, respectively. Chromatograms of the samples obtained from 50 μl of blank rat plasma (B) and blank rat bile (D) are also shown. The detection wavelength was 215 nm for rat plasma and 304 nm for rat bile, respectively. In our study of the biliary excretion of MPA, we found that more than 4.5% of the administered dose was actively recovered in the bile 6 h after dosing. The MPA concentration in rat bile was considerably higher than that in plasma and moreover, more peaks were found around the retention time of MPA. Therefore, the UV detection wavelength was shifted to 304 nm, where the contribution of background peaks was small, although the sensitivity of MPA detection decreased. As shown in the figure, no interfering peaks were detected in chromatograms of the rat plasma or bile samples obtained before the administration of MPM.

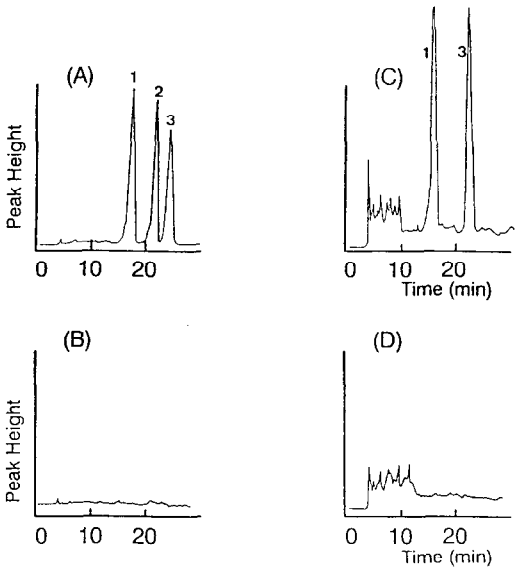

Fig. 3. Chromatograms of biological samples. (A) Rat liver homogenate spiked with MPA (13.0 μg/ml) and MPM (9.61 μg/ml); (B) blank rat liver homogenate sample; (C) human plasma spiked with MPA (2.0 μg/ml); (D) blank human plasma sample. Peaks: 1 = MPA; 2 = MPM; 3 = diazepam (internal standard).

Fig. 3 shows chromatograms of samples obtained from 0.5 ml of rat liver homogenate to which MPM and MPA were added at concentrations of 13.0 μg/ml and 9.6 μg/ml (A), respectively, and the blank sample (B). The UV detector was operated at 304 nm for the same reason as described above for the bile sample. No interfering peaks were detected in the chromatograms. Evenso, no interfering peaks were detected in the chromatograms for kidney and small intestinal epithelial cells samples. Fig. 3 also shows a chromatogram of a sample obtained from 1 ml of human plasma (C) spiked with MPA (2.0 μg/ml) and that of a sample obtained from 1 ml blank human plasma (D). In the pharmacokinetic study of MPM in organ transplant patients, it was possible to use 1 ml of plasma sample for each assay. MPA was thus detected at 304 nm on the chromatogram with high accuracy. The blank plasma sample from which the chromatogram (D) in Fig. 3 was obtained was from renal transplant patients who received other immunosuppressants such as

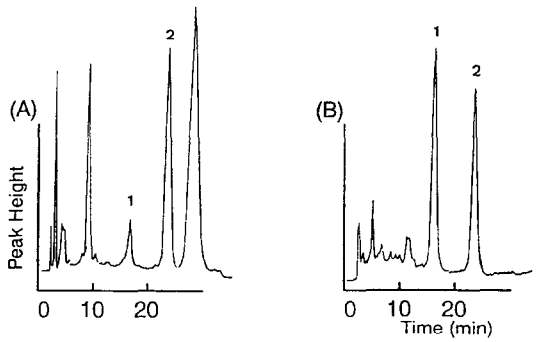

Fig. 4. Chromatograms of plasma samples taken after oral administration of MPM at doses of 16.6 mg/kg (A: rat plasma, 8 h after dosing) and 50 mg/kg (B: human plasma, 12 h after dosing). Peaks: 1 = MPA; 2 = diazepam (internal standard).

prednisolone and cyclosporin A. No interfering peaks were observed where the MPA and internal standard peaks eluted. The blank plasma samples from another 10 renal transplant patients also showed no detectable peaks that could interfere with the MPA assay. Fig. 4 shows the chromatograms of samples taken after oral administration of MPM at doses of 16.6 mg/kg (rat plasma) and 50 mg/kg (human plasma).

In these assays, MPA, MPM, diazepam and indomethacin were eluted at retention times of 16.8, 21.2, 23.5 and 36.0 min, respectively. In the assays of human plasma and rat tissue

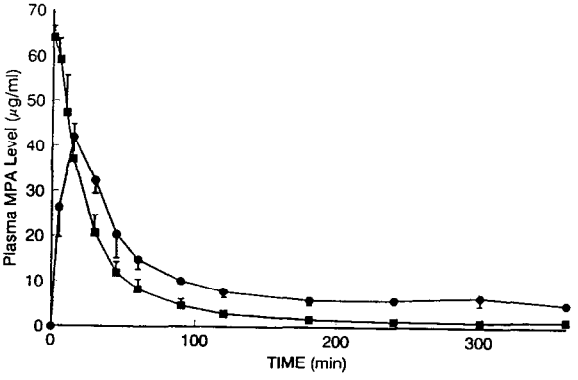

Fig. 5. Time courses of plasma MPA levels after intravenous bolus injection and intraduodenal administration of MPM to rats, at doses of 16.6 mg/kg and 50 mg/kg, respectively. (■) Intravenous bolus injection; (●) intraduodenal administration. Each value represents the mean ± S.D. of four rats.

homogenate, the HPLC system was ready for a new cycle in 25 min. In the assay of MPA in rat bile or plasma, however the HPLC system was ready for a new cycle only 60 min after injection, *i.e.* the time needed for stabilization of the baseline.

The calibration curves were linear in the ranges 0.1–100.0 μg/ml of MPA in 50 μl of rat plasma, 0.1–100.0 μg/ml of MPA in 1.0 ml of human plasma, 10.0–100.0 μg/ml of MPA in 50 μl of rat bile, and 0.8–9.6 μg/ml of MPA or 1.1–13.0 μg/ml of MPM in 0.5 ml of rat tissue homogenates, liver, kidney, and small intestinal epithelial cells. The correlation coefficient was higher than 0.999 in all calibration curves. The equations for the linear regression were as follows: rat plasma, $y = 5.58x + 0.06$; human plasma, $y = 1.38x + 0.01$; rat bile, $y = 1.17x + 0.19$; rat tissue homogenates; $y = 13.86x - 0.02$ (MPM), $y = 9.13x + 0.57$ (MPA).

Inter-assay reproducibility of the procedure was evaluated by extracting and analyzing replicate rat plasma, bile, homogenate and human plasma with known amounts of MPA and MPM. Between 8 and 30 samples were analyzed; the coefficients of variation (C.V.) are summarized in Table 1. Intra-assay reproducibility was evaluated by repeated analysis of five samples of rat plasma, bile, homogenate and human plasma containing 50 μg/ml of MPA and the internal standard. The coefficients of variation (C.V.) are summarized in Table 1. The accuracy was evaluated by the deviation of the mean value from the actual value. These results are also summarized in Table 1. Good reproducibility and accuracy were obtained in each assay.

Table 1
Reproducibility of HPLC assays of MPA and MPM

Drug concentration (μg/ml)	Inter-assay		Intra-assay		
	n^a	C.V. (%)	n^a	C.V. (%)	Mean residual (μg/ml)
Human plasma					
0.5	30	5.7	5	6.2	0.03
5.0	30	4.4	5	5.8	0.17
50.0	30	2.4	5	2.4	−0.02
Rat plasma					
0.5	30	5.2	5	4.5	0.02
5.0	30	5.3	5	2.2	−0.11
50.0	30	3.1	5	2.7	0.86
Rat bile					
10.0	10	6.1	5	8.5	0.21
50.0	10	3.8	5	5.3	1.18
100.0	10	5.4	5	6.7	3.57
Rat liver homogenate[b]					
3.2 (10 n*M*)	8	3.4	5	4.9	0.15
6.4 (20 n*M*)	8	2.7	5	3.3	−0.32
9.6 (30 n*M*) (MPM)	8	2.7	5	3.1	−0.07
4.3 (10 n*M*)	8	3.5	5	5.1	0.11
8.7 (20 n*M*)	8	2.7	5	3.7	0.24
13.0 (30 n*M*)	8	2.7	5	2.5	−0.27

[a] Number of experiments.
[b] Homogenate sample prepared by spiking MPA and MPM into rat liver homogenate.

The mean recovery and coefficients of variation from 1 ml of human plasma, from 50 μl of rat plasma or bile and from 0.5 ml of rat liver homogenate are summarized in Table 2, which reflects the accuracy and precision of this method.

The major metabolite of MPA in humans is a phenolic glucuronide. MPA glucuronide was enzymatically hydrolyzed to MPA, and the total concentration of MPA and MPA glucuronide was measured by this HPLC method. By subtracting the MPA concentration from the total concentration, we estimated the MPA glucuronide concentration. It has been reported that approximately 91% of the phenolic glucuronide of MPA was hydrolyzed within 10 min and that 100% was hydrolyzed within 30 min at pH 6.0 [6]. We found that more than 99% of the glucuronide was hydrolyzed within 30 min under our experimental conditions.

Several methods have been used for the determination of MPA, as described in the Introduction. Bopp *et al.* [6] have reported a GC method for the determination of MPA from 1 ml of human plasma. The HPLC method reported by Lee *et al.* [9] is the only method for determining MPA in biological samples such as human or monkey plasma after the administration of MPM. However, as their method requires a 5-ml sample volume to determine the plasma MPA concentration, their HPLC method is not suitable for use in pharmacokinetic studies in small experimental animals such as the rat. To overcome this problem, we developed a new HPLC method for the determination of MPA in rat plasma. To achieve a reasonably sharp peak of the drug and internal standard, the column temperature was set at 50°C. For the aqueous component of the mobile phase, 0.01 *M* phosphate buffer with an adjusted pH value of 5.0 gave the best results.

Our sample preparation procedure was based on liquid–liquid extraction. We tested three kinds of organic solvents, chloroform, ethyl acetate and dichloromethane, for the extraction of MPA. When chloroform was used, the greatest extraction efficiency was obtained. Precipitating proteins by adding methanol before the drugs were extracted into chloroform enabled us to aspirate the aqueous phase easily.

In case of purification of rat bile samples, many interfering peaks were detected in the chromatogram; therefore, MPA in the aqueous phase was washed with two kinds of organic solvents prior to the extraction as described in the previous section.

Our HPLC method has four advantages: (1) it allows the performance of pharmacokinetic studies of MPM in small experimental animals such as the rat; (2) since the extraction procedure is relatively simple, the method is suitable for measuring many types of laboratory and clinical samples; (3) it has a high specificity, since there is no influence of other immunosuppressants that may be used in combination with MPM, as described above; (4) both MPM

Table 2
Extraction efficiency from rat and human body fluids spiked with MPA and MPM

Sample	Drug concentration (μg/ml)	Recovery			Absolute recovery		
		Mean (%)	n^a	C.V. (%)	Mean (%)	n^a	C.V. (%)
Human plasma	50.0	103.1	9	5.1	51.9	3	6.1
Rat plasma	50.0	102.7	9	4.8	72.6	3	3.8
Rat bile	50.0	95.1	6	3.9	44.2	3	8.2
Rat liver homogenate[b]	9.6 (30 n*M*) (MPM)	101.1	6	3.6	55.1	3	6.6
	13.0 (30 n*M*)	98.3	6	4.3	51.2	3	7.4

[a] Number of experiments.
[b] Homogenate sample prepared by spiking MPA and MPM into rat liver homogenate.

and MPA can be determined on the same chromatogram. As the difference between the retention times of MPM and MPA is 5.0 min, there is no problem in performing a routine analysis in the laboratory.

Our assay method has been used successfully for the analysis of more than 1000 samples. Using this method, we have performed both clinical pharmacokinetic studies of MPM in renal transplant patients and basic pharmacokinetic studies in experimental animals such as the rat. Through such studies, we have obtained results that elucidate the pharmacokinetic characteristics of this new immunosuppressant. Fig. 5 shows the results of a pharmacokinetic study performed in rats that received either an intravenous (i.v.) bolus injection or intraduodenal (i.d.) administration of MPM at doses of 16.6 mg/kg and 50 mg/kg, respectively. MPM was not detectable even at 1 min after i.v. bolus injection, suggesting that this agent is rapidly hydrolyzed to MPA in the plasma. The plasma MPA level declined biexponentially. We have also used this assay method in biopharmaceutical studies in the rat with the aim to determine the stability of MPM in the gastrointestinal tract, the biliary excretory characteristics of the drug and the enzymatic conversion from MPM to MPA. Further, to investigate the relationship between the immunosuppressive activity of MPM, and its plasma levels, we have also been examining a rat heart transplant model to determine whether graft survival is prolonged by orally administered MPM.

4. Acknowledgments

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