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STABILITY AND PHARMACOKINETIC STUDIES OF A NEW IMMUNOSUPPRESSANT, MYCOPHENOLATE MOFETIL (RS-61443), IN RATS

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ABSTRACT

Mycophenolate mofetil (MPM), a new immunosuppressant, is a morpholinoethyl ester of mycophenolic acid (MPA). The enzymatic and non-enzymatic hydrolysis was studied in an artificial digestive fluid, rat plasma, and tissue homogenates. MPM was chemically stable in the artificial digestive fluid. In rat tissue homogenates and plasma, MPM was rapidly hydrolysed to MPA. The conversion rate of MPM to MPA in various rat tissue homogenates was in the order of liver > kidney > plasma > small-intestinal epithelial cells. After the intravenous injection of MPM at 16·7 mg kg^{-1}, the terminal elimination half-life, $t_{1/2\beta}$, was 4·74 ± 0·33 (mean ± SD) h, and the area under the plasma concentration versus time curve, AUC, was 48·78 ± 6·01 μg h mL^{-1}. After intraduodenal (ID) administration of MPM at 16·7 mg kg^{-1}, $t_{1/2\beta}$ was 3·92 ± 1·05 h, and the AUC was 38·08 ± 8·30 μg h mL^{-1}. The systemic availability of MPA after ID MPM dosing was 1·52 times higher than that after ID administration of MPA. This result supports the usefulness of MPM as an oral prodrug of MPA as a new oral immunosuppressant.

KEY WORDS: mycophenolate mofetil; mycophenolic acid; prodrug; pharmacokinetics; rat

INTRODUCTION

Mycophenolate mofetil (MPM), a new immunosuppressant, is a morpholinoethyl ester of mycophenolic acid (MPA), which is its active metabolite. MPA, a fermentation product of several *Penicillium* species, is a potent, non-competitive, 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] In a study of immunosuppressive

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activity, MPM has been shown to prolong heart, renal, and islet allograft survivals in rats, mice, and dogs.[3–5] Clinical studies of MPM in human organ transplant recipients are now in progress.[6,7] In a study of pharmacokinetics, Lee *et al.*[8] performed a comparative bioavailability study of MPM and MPA in monkeys. The area under the plasma MPA concentration versus time curves (AUC) obtained after MPM administration was about 1·5 times higher than that of MPA. The bioavailability of MPA was significantly improved by administration of MPM as a prodrug of MPA. In a primate cardiac allograft model, the AUC obtained after oral administration of MPM has been reported to be related to the dose of MPM.[9,10] Yatscoff *et al.*[11] performed a pharmacokinetic study of MPM in rabbits following IV and SC administrations of MPM. However, there have been no detailed studies on the metabolic fate of MPM as a prodrug of MPA, namely, enzymatic hydrolysis in several tissues and the stability of MPM in the gastrointestinal tract, and a basic pharmacokinetic study in rats.

Previously, we reported a new selective, sensitive, and simple assay method for both MPM and MPA in rat plasma, bile, and tissue homogenates and in human plasma by high-performance liquid chromatography (HPLC).[12] This method enables a pharmacokinetic study in small experimental animals. Using this method to elucidate the metabolic behaviour in the gastrointestinal tract, we examined the enzymatic conversion of MPM to MPA in rat tissue homogenate and the stability of MPM in artificial digestive fluid. In addition, a basic pharmacokinetic study on MPM and MPA has been performed in rats.

MATERIALS AND METHODS

Chemicals and reagents

MPM was kindly supplied by Syntex Research (Palo Alto, CA, U.S.A.). MPA was purchased from Sigma (St. Louis, MO, U.S.A.). Reagent grades of pepsin, pancreatin, and polyethylene glycol 400 were obtained from Nacalai Tesque (Kyoto, Japan). All other reagents were of reagent grade.

Preparation of the artificial digestive fluid

The artificial gastric fluid was prepared by mixing 24 mL of 10% (v/v) hydrochloric acid, 2·0 g of NaCl, and distilled water to the final volume of 1000 mL (solution A). The artificial intestinal fluid was also prepared by mixing 250 mL of 0·02 M potassium dihydrogenphosphate solution, 118 mL of 0·2 N sodium hydroxide, and distilled water finally to 1000 mL (solution B). The pH values of solutions A and B were 1·2 and 6·8, respectively. In addition, solution C was prepared by adding 10 g of pepsin to 1000 mL of solution A. Solution D was prepared by adding 10 g of pancreatin to 1000 mL of solution B.

Preparation of rat tissue homogenates

Male Wistar rats each weighing 300–350 g were used. The animals were killed by cervical dislocation, and their livers and kidneys were perfused with ice cold 0·05 M Tris-HCl buffer (pH 7·4) containing 1·15% KCl). To 1 g of liver or kidney sample, 4 mL of the same ice cold solution was added, and the mixture was homogenized with a glass homogenizer. Epithelial cells from the small intestine, obtained by scraping with a cover glass, were directly homogenized with a fourfold volume of the same ice cold solution with a glass homogenizer. The supernatant fraction obtained after the removal of the cell debris by centrifugation at 1000*g* for 5 min was immediately used for the hydrolysis experiment. The average protein concentration of each homogenate was determined by the method of Markwell *et al.*[13]

The hydrolysis study

In the artificial digestive fluid, solutions A and B were used as the chemical media for the hydrolysis study of MPM. Solution C, containing pepsin, and solution D, containing pancreatin, were used for the enzymatic hydrolysis study. The stock solution of MPM in methanol (10 μL) was added to 10 mL of solutions A, B, C, and D to start the reaction after preincubation for 5 min at 37 °C, and the concentration of MPM in each solution was finally adjusted to 30 μM. After incubation under blowing air at 37 °C with shaking, 0·5 mL of the incubation medium was withdrawn at 15, 30, 60, and 120 min, and the obtained sample was mixed with 1 mL of methanol to stop the reaction at each sampling time.

In plasma, the blood was taken from male Wistar rats each weighing 350–400 g using a heparinized centrifuge tube and the plasma fraction was obtained by centrifugation at 3000 rpm for 10 min. Ten microlitres of the stock MPM methanol solution was added to 5 mL of plasma to start the reaction after preincubation for 5 min at 37 °C, and the concentration of MPM in plasma was finally adjusted to 30 μM. After incubation under the air blow at 37 °C with shaking, 0·5 mL of the incubation mixture was withdrawn at 2, 5, 10, and 20 min, and immediately mixed with 1 mL of ice cold methanol to stop the reaction at each sampling time. The average protein concentrations of plasma were determined by the method of Markwell *et al.*[13]

In tissue homogenate, the incubation mixture (10 mL) was prepared by adding the tissue homogenate to 0·1 M Tris-HCl buffer (pH 7·4) containing MPM as the methanol solution. The final concentration of MPM was 30 μM, and the homogenate was diluted finally to 2% (w/v). The mixture without homogenate was preincubated for 5 min at 37 °C, and the reaction was started by the addition of tissue homogenate. After incubation under blowing air at 37 °C with shaking, 0·5 mL of the incubation mixture was withdrawn at 2, 5, 10, and 20 min and immediately mixed with 1 mL of ice cold methanol to stop

the reaction at each sampling time. In addition, to examine the enzymatic activity of plasma under the same condition, we used the diluted rat plasma (2%) prepared according to the method for the tissue homogenate as described above as the incubation mixture. The concentrations of MPM and MPA in each sample were determined by an HPLC procedure as described below. The values are expressed as the mean values ± SD.

The pharmacokinetic study

Male Wistar rats weighing 350–400 g were used. The rats were fasted overnight but had free access to water. Under anaesthesia by an intraperitoneal injection of sodium pentobarbital, 32 mg kg^{-1}, a polyethylene cannula (ID 0·5 mm, OD 0·8 mm; Dural Plastics, Australia) was surgically introduced into the left carotid artery to obtain blood samples at various times. Between the samplings, the cannula was filled with heparinized saline.

The drug solutions for all administration routes were prepared by dissolving MPM and MPA in polyethylene glycol 400 (PEG 400) and stored at 4 °C until use. The concentration of the test solution for the IV study was 16·7 (MPM) and the volume of injection was 1 mL kg^{-1}. For the ID study, the concentrations of the test solution were 8·4 (MPM) and 6·2 (MPA) mg mL^{-1} and the volume of injection was 2 mL kg^{-1}.

Twenty rats were divided into five groups, A–E. After collecting blank blood samples, MPM solutions were intravenously administered to group A rats at a dose of 16·7 mg kg^{-1}. In group B rats, the MPM solution was injected into the duodenum at a dose of 16·7 mg kg^{-1}. In group C rats, the MPA solution was injected into the duodenum at 12·3 mg kg^{-1}, which is equivalent to 16·7 mg kg^{-1} of MPM. In group A–C rats, blood samples (120 μL) were drawn into the heparinized microcentrifuge tubes at times 5, 10, 15, 20, 30, 60, 90, 120, 180, 240, 300, and 360 min after drug administration. In group D rats, MPM solution was injected into the duodenum at 50·0 mg kg^{-1}. In group E rats, to examine the absorption of MPM from the stomach, the MPM solution was administered at a dose of 50 mg kg^{-1} into the stomach of which the pylorus and cardia were ligated. In group D and E rats, blood samples (120 μL) were withdrawn into heparinized microcentrifuge tubes at times 5, 10, 15, 20, 30, 60, 90, 120, and 180 min after drug administration. All the blood samples were immediately centrifuged to obtain the plasma fraction (50 μL) and were stored at −20 °C until analysis.

In groups D and E, the area under the plasma concentration–time curves (AUC) after ID and intrastomach (IS) administration (50 mg kg^{-1}) were calculated using the trapezoidal rule up to the last measured plasma concentration (3 h after dosing). In group A–C rats, the terminal elimination rate constant, β, for the plasma MPA concentration–time curves after IV and ID administration was determined by linear regression analysis of at least three data points from the terminal portion of the plasma concentration–time plots.

The AUCs after IV and ID administrations were calculated using the trapezoidal rule up to the last measured plasma concentration, $C_{p(last)}$, and extrapolated to infinity by addition of the correction term $C_{p(last)}/\beta$. The terminal elimination half-life, $t_{1/2\beta}$, was determined by dividing ln 2 by β. The total plasma clearance, CL_{tot}, was determined by dividing the dose by the AUC value obtained after IV administration. The parameters were determined for each individual. The values are expressed as the mean ± SD. Differences with $p < 0{\cdot}05$ were considered to be significant (two-sided t test).

Sample treatment and the HPLC assay

The concentration of MPA was determined by an HPLC procedure previously reported from our laboratory.[12] The preparation of samples was based on liquid–liquid extraction with chloroform. 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 a wavelength of 215 nm for rat plasma and 304 nm for tissue homogenate. 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 mm × 6·0 mm ID, 5 μm). Samples were injected with an SIL-10A automatic injector. The system was controlled with an SCL-10A system controller. The area under each peak was calibrated with a Shimadzu data processor (CR-5A Chromatopac). Levels were estimated by a chromatographic technique using comparison of the peak areas obtained from samples containing a known amount of MPM and MPA for standard curves. The standard curves of MPM and MPA were linear over the range of 0·1–150 μg mL^{-1}. The CV values (%) of our inter- and intra- assay reproducibilities for rat plasma were 3·1–5·2% and 2·7–4·5% ($n = 30$), respectively.

RESULTS

The hydrolysis study

The non-enzymatic hydrolysis of MPM was examined in the artificial digestive fluids solution A (pH 1·2) and solution B (pH 6·8). In solution A, hydrolysis of MPM was not observed after incubation for 120 min at 37 °C. In solution B, only 3·25% of MPM was converted to MPA within 120 min.

The enzymatic hydrolysis of MPM was also examined in the artificial digestive fluids solution C (pH 1·2, containing pepsin) and solution D (pH 6·8, containing pancreatin). As shown in Figure 1, although the conversion of MPM to MPA was not observed in solution C, 35·3% of MPM was converted to MPA within 120 min in solution D. In both the enzymatic and non-enzymatic hydrolyses in artificial digestive fluids, MPM was shown to be stable.

On the other hand, 50·7% of MPM was hydrolysed to MPA within 2 min and 97·3% was hydrolysed within 10 min in rat plasma, where the average protein concentration was 69·4 mg mL^{-1}. Next, the stability of MPM was studied in diluted rat plasma and tissue homogenates (Figure 2). The average protein concentrations of plasma and homogenates of kidney, liver, and small-intestinal epithelial cells were 1·16, 1·76, 2·57, and 1·88 mg mL^{-1}, respectively. In the liver homogenate, MPM was completely hydrolysed to MPA within 2 min. In the kidney homogenate, the conversion rate of MPM to MPA was

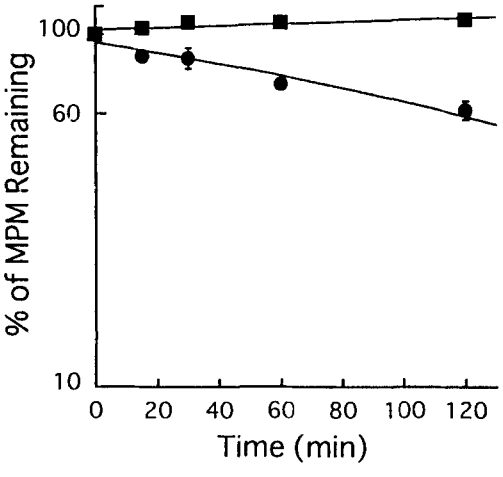

Figure 1. Hydrolysis of MPM in the artificial digestive fluid: ■, solution C (containing pepsin); ●, solution D (containing pancreatin). Each value represents the mean ± SD of four experiments

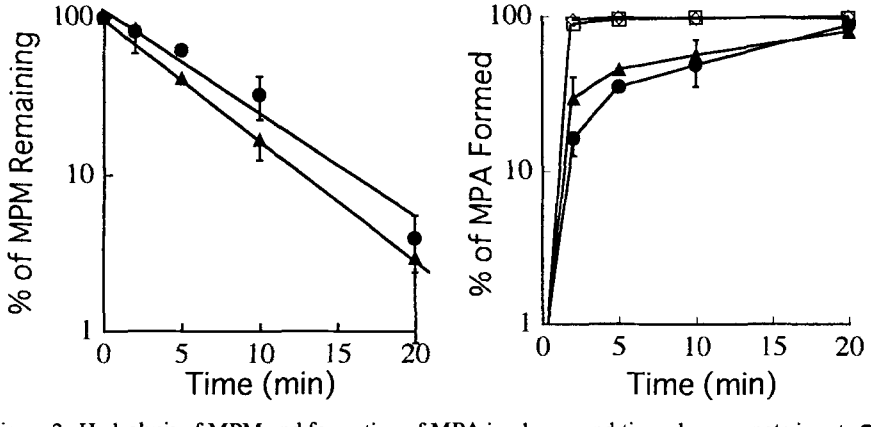

Figure 2. Hydrolysis of MPM and formation of MPA in plasma and tissue homogenate in rat: ●, diluted plasma; ▲, epithelial cells from small intestine; □, kidney; ◇, liver. Each value represents the mean ± SD of four experiments

94% within 2 min and 100% within 5 min. The first-order hydrolysis rate constant of MPM was calculated by linear regression analysis of a logarithmic plot of remaining drug concentration versus time. In the kidney homogenate, the hydrolysis rate constant was 1·36 min^{-1}. In both cases, the conversion rates were extremely rapid. In diluted plasma and epithelial cells from the small-intestinal homogenate, the hydrolysis rate constant was 0·172 and 0·183 min^{-1}, respectively.

The pharmacokinetic study

Figure 3 shows the mean plasma MPA concentration–time profiles after IV and ID administrations of MPM or MPA to rats. The pharmacokinetic parameters for these groups of rats are shown in Table 1. The plasma MPA levels plotted on a semilogarithmic scale clearly revealed that MPA disappeared from the systemic circulation in a two-exponential decay after IV bolus injection of MPM. MPM was not detectable even at 1 min after IV injection of MPM. The systemic availability (SA) of MPA from MPM was determined by comparing the mean values of the AUC obtained after ID MPM administration with that after IV MPM administration. The mean SA of MPA

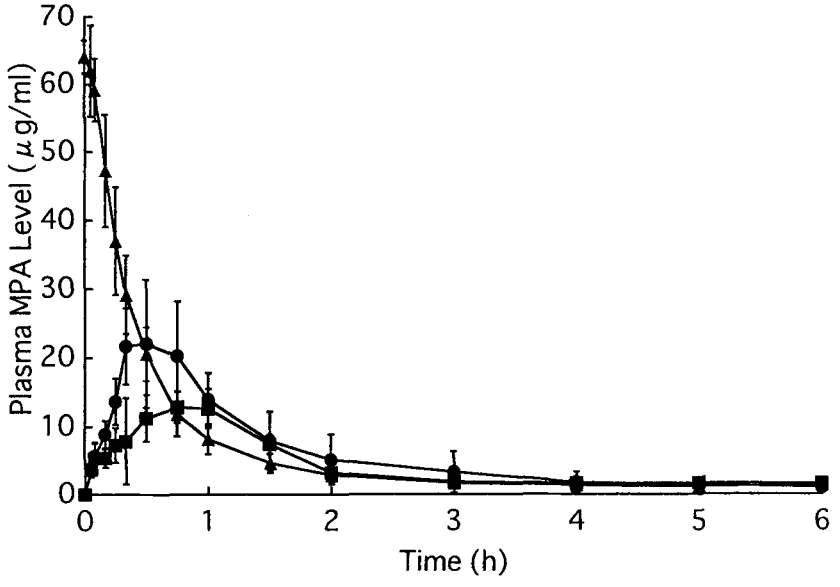

Figure 3. Time courses of plasma MPA levels after intravenous bolus injection and intraduodenal administration of MPM or MPA to rats: ▲, intravenous bolus injection of MPM at a dose of 16·7 mg kg^{-1}; ●, intraduodenal administration of MPM at a dose of 16·7 mg kg^{-1}; ■ intraduodenal administration of MPA at a dose of 12·3 mg kg^{-1} (equivalent to 16·7 mg kg^{-1} of MPM). Each value represents the mean ± SD of four rats

Table 1. Pharmacokinetic parameters of MPA obtained after IV and ID administration of MPM and MPA to rats. Each value represents the mean ± SD

Pharmacokinetic parameters	Rat group		
	Group A (MPM, IV)	Group B (MPM, ID)	Group C (MPA, ID)
Dose ($mg\,kg^{-1}$)	16·7	16·7	12·3 (MPA)
CL ($L\,h^{-1}$)	1·78 ± 0·20	2·19 ± 0·42	2·83 ± 0·31
$t_{1/2\beta}$ (h)	4·74 ± 0·33	3·92 ± 1·05	3·85 ± 0·47
AUC ($\mu g\,h\,mL^{-1}$)	48·78 ± 6·01	38·08 ± 8·30	25·71 ± 5·20

after administration of MPM as a prodrug was 78·1%. Because MPM is a morpholinoethyl ester prodrug of MPA, the improvement in SA was evaluated by comparison of the mean values of the AUC obtained after ID MPA administration with that of ID MPM administration. About a 1·52-fold higher AUC was obtained after ID MPM administration than after ID MPA administration. As shown in Figure 3, the mean peak plasma MPA level was 22·1 ± 9·3 $\mu g\,mL^{-1}$ (mean peak time was 30 min) for ID MPM administration and 12·9 ± 2·2 $\mu g\,mL^{-1}$ (mean peak time was 45 min) for ID MPA administration.

Table 2 shows the values of AUC_{0-3} obtained after ID and IS administrations of MPM. The amount of drug absorbed from the intestine in 3 h was 8·13 times higher than that from the stomach of which the pylorus and cardia were ligated.

DISCUSSION

MPM is an ester-type prodrug of MPA to improve the SA of MPA. It is indispensable for MPM to retain its chemical form up to the absorption site of this drug after oral administration. Therefore, this kind of prodrug requires stability in the gastrointestinal tract. Although many investigators used buffered solutions with various pH values as the chemical media for the hydrolysis study, we used an aritifical digestive fluid, which is described in

Table 2. The AUC value of MPA obtained after ID and IS administration of MPM to rats. Each value represents the mean ± SD

	Rat group	
	Group D (ID)	Group R (IS)
Dose ($mg\,kg^{-1}$)	50·0	50·0
AUC_{0-3} ($\mu g\,h\,mL^{-1}$)	44·7 ± 3·2	5·5 ± 0·6

Japanese pharmacopoeia. In a non-enzymatic hydrolysis study, the hydrolysis of MPM was not observed, suggesting that MPM is chemically stable in the digestive fluid. However, the hydrolysis of MPM was observed in solution D (35·3% of MPM was hydrolysed within 120 min). These findings suggest that a small amount of MPM would be hydrolysed in the gastrointestinal tract. However, because this drug was rapidly absorbed from the rat intestine as described above, probably only a small amount of MPM is hydrolysed by the digestive fluid before absorption.

Carboxylesterase is distributed in many kinds of tissue. The activation of this ester-type prodrug of MPA is ascribed to the hydrolytic action of this enzyme in the blood, liver, and intestine. In the present study, the hydrolysis of MPM in rat plasma was inhibited by the addition of bis(*p*-nitrophenyl) phosphate, which is an inhibitor of carboxylesterase[14] (data not shown). Hydrolytic activities in plasma show a variability among species.[15,16] Carboxylesterases in human and rat plasma have been reported to be different proteins.[17,18] The rat carboxylesterase has a molecular weight of 72 kDa and no subunits. However, the human one is composed of four subunits and has a molecular weight of 320 kDa. On the other hand, the carboxylesterases in human and rat liver are homogeneous, and the same amino-acid sequence can be found around the serine residue in the active site of the enzymes.[19] It has been reported that the times for MPM to reach 50% conversion to MPA in human plasma, monkey plasma, and mouse liver homogenate were 3·5 h, 8·3 h, and < 5 s, respectively.[8] However, in our experiment, the hydrolysis of MPM in rat plasma was extremely rapid, and the hydrolysis rates in rat small-intestinal epithelial cells and plasma were approximately the same. In the rat liver homogenate, MPM was immediately hydrolysed. In addition, because we used diluted homogenates (2% w/v) for these experiments, the conversion rate of MPM to MPA would be very rapid in the *in vivo* situation. These results suggest that the hydrolysis progressed as soon as MPM was absorbed from the rat small intestine, and the conversion of MPM to MPA would be completed by the first-pass effect of the liver etc. The results of the hydrolysis study in rat liver and kidney homogenates may not represent the *in vivo* situation. Though the conversion rate of MPM to MPA in human plasma was slower than that in rat plasma, MPM would be immediately hydrolysed in both rat and human livers. In addition, MPM was not detectable at 30 min after oral administration of MPM in human plasma, though the data are not shown in this report. Therefore, it is presumed that the conversion of MPM to MPA in humans would also be completed by the first-pass effect of the liver etc.

Our sensitive HPLC assay method enabled the basic pharmacokinetic study in small experimental animals. Using this method, we have obtained results that elucidate the pharmacokinetic characteristics of this new immunosuppressant in the rat. The dose of MPM to be used for IV or ID administration was determined using the results of our pharmacodynamic study in which the relationship between the prolongation of graft survival by

this drug and its plasma levels was elucidated. In the IV study, MPM was not detectable even at 1 min after IV bolus injection, suggesting that this drug is rapidly hydrolysed to MPA in the blood circulation. This is in accordance with the results of the hydrolysis study in rat plasma described above. It has been reported that the plasma concentration versus time data for MPA in rabbits following administration of MPM via the IV route were fitted to a two-compartment open model.[11] In our experiment in rats, the plasma MPA level declined biexponentially after IV bolus injection. Lee *et al.* reported that the AUC after oral administration of MPM in cynomolgus monkeys at a dose of 20 mg kg^{-1} using the total MPA (MPA and MPA glucuronide) concentration in plasma was about 1·5 times higher than that obtained after oral MPA administration as described in the introduction.[8] In our SA study evaluating the plasma free MPA levels after ID administration of MPM or MPA in rats, we also obtained the same result (AUC after ID administration of MPM to rat was 1·52 times higher than that of ID-administered MPA).

We determined the SA of MPM from the stomach under the condition of ligation of both the pylorus and cardia. The value of AUC_{0-3} after IS administration of MPM (50 mg kg^{-1}) was 5·5 μg h mL^{-1}. Only a small amount of MPM was absorbed from the stomach in this experiment. The value of AUC_{0-3} after ID administration of the same dose of MPM was 44·7 μg h mL^{-1}. Moreover, because the gastric emptying time is so short, as described above, little MPM would be absorbed from the stomach after oral administration of MPM to the experimental animal.

In this study, we reported the enzymatic and non-enzymatic hydrolyses of MPM and pharmacokinetic properties of MPM in the rat. We conclude that MPM exhibits the following characteristics in the rat: (i) MPM is enzymatically and non-enzymatically stable in the digestive fluid; (ii) MPM was rapidly hydrolysed in rat plasma and tissue homogenates, and the conversion rates of MPM to MPA in different rat tissues were in the order of liver > kidney > plasma > small-intestinal epithelial cells; (iii) the plasma MPA level declined biexponentially after IV administration of MPM; (iv) MPM was rapidly absorbed after ID administration of MPM (mean peak time was 30 min); (v) the mean SA of MPA after administration as MPM was estimated to be 78·4%; (vi) MPM, which is developed as a lipophilic ester of MPA for improving the SA of MPA, would be efficiently converted to MPA after rapid absorption from the gastrointestinal tract (the SA of MPA after ID administration as MPM was 1·52 times higher than that after ID MPA administration); (vii) it was thought that little MPM was absorbed from the stomach.

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