CHAPTER III

PATIENT AND METHOD

Patient and method

The study was conducted from August 2001 to December 2002 at Rajavithi Hospital, Bangkok, Thailand.

Patient

Study population

This study was designed as a prospective unicenter trial to evaluate relationship between MPA plasma concentrations at various times including area under the plasma concentration-time curve (AUC) to gastrointestinal adverse event (GAE) of MMF in renal transplanted Thai patients. The study protocol was reviewed and approved by the ethic committee of Rajavithi Hospital. The subjects of this study were selected from a group of the post-renal transplanted patients who came to follow up at the medicine department, renal clinic, Rajavithi Hospital. All patients received a triple immunosuppressive regimen (MMF, cyclosporin, and prednisolone). Written informed consent had to be given by the patients. Patients were fasting after midnight until the blood samples were drawn in the morning 1 hour after MMF administration. The criteria for enrollment were as followed :

Inclusion criteria

The patients who had all of these characteristics were enrolled in this study.

1. Post-renal transplanted patients at time over 1 month. The patients were directly from Rajavithi Hospital or referred from other hospitals and came to follow up at the medicine department, renal clinic, Rajavithi Hospital.

2. The patients were going to start or during treatment with MMF.
3. The patients knew details and consented to enroll in this study.

Exclusion criteria [13, 15, 28, 31]

The patients who had either one of these characteristics were excluded from this study.

1. The patients had experienced either severe gastrointestinal disorders and / or severe diarrhea such as watery stool over all more than 5 times daily which was not causing by MMF since this might change the pharmacokinetic properties of MMF .
2. The patients had active peptic ulcer during this study.
3. The patients or the donors had serologic evidence of leukemia T-cell type, Human Immunodeficiency virus (HIV) or Hepatitis B or C.
4. The patients had symptoms of malignancies.
5. The patients had systemic infections which must be treated during this study.
6. The patients had white blood cell count less than 2,500 cells/mm^3 or hemoglobin less than 5 gm/deciliter or platelet count less than 100,000 cells/mm^3
7. The patients had concomitant therapy, which might change the pharmacokinetic properties of MMF such as probenecid, cholestyramine, magnesium-or aluminium hydroxide-containing antacids, sodium salicylates, ferrous containing medications such as ferrous sulfate and tacrolimus or the other drugs which might strongly change blood level of MPA.
8. The patients had hepatitis disease or hypoalbuminemia (serum albumin less than 3 gm/dL)
9. The patients had severe chronic renal dysfunctions (serum creatinine clearance less than 20 mL/min.)
10. Pregnancy or lactation patients
11. The patients had known allergy or history of allergy to MMF
12. The patients could not be followed up throughout this study.
13. The patients were diagnosed from physicians to be inappropriate to enroll in this study.

Method

Study design and sample collection

Twenty-five post-renal transplanted patients who met the aforementioned criteria were participated in this study. The transplanted kidneys were provided from cadaver or living donors.

Patients had already started MMF treatment (during treatment)

Blood samples to create complete plasma concentration-time profiles (9 points) were collected at steady state after the patients who enrolled in the study has been administered with their current dosage regimen. Blood samples were collected at 0, 0.25, 0.5, 1, 2, 3, 4, 6 and 12 hours after MMF administration. The dosage regimen were increased 500 mg daily a time for one week (if no GAE occurred, MPA AUC reference range or 2 gm daily dose had not been reached). When MPA AUC were higher than the reference range, the dosage regimen were decreased 500 mg daily. Each time, 4 - 6 blood samples were again collected at 0, 1, 3 and 6 hours or 0, 0.5, 1, 3, 4 and 6 hours after MMF administration (the sampling times were at first designed according to the work of Willis C, et al[19] which required only 4 points. Later on, from our study, we found that C peak were showed up equally often at C0.5 and C1, also, the secondary peak was often showed up at C4, we therefore added two more sampling times at 0.5 and 4 hours after MMF administration for the later cases). Step of the study was demonstrated in figure 1 (A-C).

Patients start MMF for the first time

The initial dose of MMF might either be 1 gm or 1.5 gm daily depend on the condition of the patients (as directed by the physician). The complete plasma sample profiles (9 points) were collected at 0, 0.25, 0.5, 1, 2, 3, 4, 6 and 12 hours after MMF administration. The dosage regimens were increased 500 mg daily a time for one week (if no GAE occurred, MPA AUC reference range had not been reached or MMF 2 gm daily dose had not been reached). Each time, 4 - 6 blood samples were again collected at

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The blood samples were put in the EDTA tubes. After the blood samples were centrifuged for 10 min at 1500 rpm then the plasma samples were separated and kept at – 70°C until analyzed.

Material

1.Apparatus and Instruments

-High performance liquid chromatograph, consists of

- LC-10AD Liquid Chromatograph (Shimadzu, Japan)
- SPD-10A UV-VIS detector (Shimadzu, Japan)
- Chromatopac C – R6A (Shimadzu, Japan)
- Injection system, Rheodyne 7167 equipped with a 20 μL loop
- Column, Symmetry-C18 (Waters, U.S.A.)
- Column, Spherisorb, 5 um, ODS2, 4.6 x 250 mm I.D. (Waters, U.S.A.)

-Analytical balance, Sartorius BA2105 (Sartorious AG, Germany)

-Microcentrifuge, Z 230 MA (Berthold Hermle AG, Germany)

-Sonicator, D-78224 (Elma, Germany)

-Vortex mixer, Genie 2 (Genie Scientific, U.S.A.)

-Nylon filter membrane, 0.45 μm (Alltect, U.S.A.)

-Micro-syringe fixed needle for rheodyne 100 μL (ITO corporation, Japan)

-Refrigerator – 70 Degree Celsius

-Blood sampling set such as syringe 5 mL, 21G needle, 20G cathelons, injection plug, normal saline, heparin 5,000 IU, EDTA tube, microtube, gauze, micropore and alcohol

2. Chemicals and Reagents

-Mycophenolate mofetil (CellCept®) 250 mg per capsule (Roche, Switzerland)
-MPA (RS-5797-000 : (E)-6-(1,3-dihydro-4- hydrox-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-4-methyl-4- hexenoate) (Roche, Switzerland)
-RS-60461-000: (E)-6-(1,3-dihydro-4-(4-carboxy-butoxy)-6-methoxy-7-methyl-3-oxo-5-isobenzo-furanyl-4-methyl-4-hexenoic acid) (Roche, Switzerland)
-Acetonitrile HPLC grade (Lab-Scan, Thailand)
-Methanol HPLC grade (Lab-Scan, Thailand)
-Phosphoric acid (H_3PO_4) (Merck, Germany)
-Potassium dihydrogen phosphate (KH_2PO_4) (Merck, Germany)

Quantitative analysis of MPA by HPLC

1.Preparation of the stock solution

Stock standard solutions of MPA (0.5 mg/mL in methanol) were prepared. Then 0.01 mL, 0.04 mL, 0.1 mL, 0.2 mL, 0.4 mL, 0.6 mL, 1.2 mL and 1.6 mL of each stock standard solution were transferred into 10 mL volumetric flask and diluted with medium. The final concentrations of MPA in standard solution were 0.5, 2, 5, 10, 20, 30, 60, 80 mcg/mL. Concentration of internal standard stock solution was 0.4 mg/mL

2. Preparation of the mobile phase

Mobile phase consist of acetonitrile and 0.1 M phosphate buffer pH 3.2 (50:50 v/v). Preparation of the 0.1 M phosphate buffer by weight potassium dihydrogen phosphate (KH_2PO_4) 12.249 gm and measure phosphoric acid (H_3PO_4) 573 mcL, both were mixed in ultra-pure water 1000 mL and adjusted to pH 3.2. Filtered through a 0.45 μm membrane filter and ultrasonically degassed after mixing.

3. Preparation of the pooled blank plasma

The pooled blank plasma was prepared from plasma sample of 6 normal subjects. Each blank sample was tested for interference by injected to HPLC. When blank plasma was discovered the interference, then was separated. All blank plasmas were mixed as the pooled blank plasma and were frozen until analyzed.

4.The standard curve of MPA in the pooled blank plasma

Standard MPA at various concentrations 0.5, 2, 5, 10, 20, 30, 60, 80 mcg/mL were added to the pooled blank plasma in order to make the standard curve. RS-60461-000 : (E)-6-(1, 3-dihydro-4-94-carboxy-butoxy)-6-methoxy-7-methyl-3-oxo-5-isobenzo-furanyl-4-methyl-4-hexenoic acid was internal standard (IS). After several steps of sample preparation according to the modified method of Na-Bangchang K, et al.[23] and Hosotsubo H, et al.[24] as show in figure 2, the samples were injected into HPLC.

The standard curve was created from the plot between MPA concentrations versus peak area ratios of MPA and internal standard.

Analytical method validation [40]

The method validation was performed according to the guidance for Industry Bioanalytical Method Validation (CDER and CVM, U.S. FDA, 2001)

1. Linearity

Linearity was tested by analyzing plasma calibration standards containing known (spiked) amounts of MPA at concentrations of 0.5, 2, 5, 10, 20, 30, 60, 80 mcg/mL. Plotted the peak area ratios of MPA and internal standard versus the concentration.

2. Accuracy

Accuracy was determined in term of the absolute recovery. The absolute recovery of MPA was assessed at the three sets of quality control samples (low, medium and high, 5 replicates at each concentration) by comparing the peak areas after sample preparation with the mean peak area obtained from direct injection of the stock solution used to spiked the samples.

3. Precision

3.1 Within-run precision

Within run precision was determined by analyzing four sets of quality control samples (2, 20, 40, 80 mcg/mL, 5 replicates at each concentration) in the same day. The percent coefficient of variation (% CV) of estimated concentration of MPA of each concentration was determined.

3.2 Between-run precision

Between run precision was determined by comparing the estimated concentration of MPA of four sets of quality control samples (2, 20, 40, 80 mcg/mL, 5 replicates at each concentration) for five different days. The percent coefficient of variation (% CV) of each concentrations was determined.

Acceptance criteria: [40]

For accuracy, the percent analytical recovery should be within $\pm$ 15 % while the percent coefficient of variation (%CV) for both the within run and between run at each concentration level should not exceed 15 % except for the lower limit of quantification (LLOQ), where it should not exceed 20 %.

4. Specificity and selectivity

For selectivity, analyses of blank samples from plasma sample of 6 normal subjects. Each blank sample was tested for interference. Comparison of retention time of MPA and internal standard in standard solution and in pooled blank plasma.

5. Preparation of the plasma samples

Concentrations of MPA in the plasma samples were quantified using the high-performance liquid chromatography (HPLC). (E)-6-(1, 3-dihydro-4-(4-carboxy-butoxy)-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-4-methyl-4-hexenoic acid was used as the internal standard (IS). Preparation of the plasma samples was modified from the method of Na-Bangchang K, et al[23]. and Hosotsubo H, et al[24]. Preparation method of the plasma samples was shown in figure 2.

Chromatographic conditions

The column was spherisorb C18 (Waters, U.S.A.). The column effluent was monitored by UV detection at 249 nm. The mobile phase was acetonitrile: 0.1 M pH 3.2 phosphate buffer (50:50 v/v). The mobile phase was filtered through a 0.45 μm pore size membrane filter prior to mixing and ultrasonically degassed after mixing. The flow rate was 0.8 ml/min. Chromatography was performed at ambient temperature.

Pharmacokinetic parameters

The AUC for time 0-12 hr (AUC_{0-12}) was calculated at steady state, using the linear trapezoidal rule up to the last measured plasma concentration drawn 12 hr after MMF administration. The minimum plasma concentrations, the maximum plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) were directly observed and determined from the plasma concentration–time curves.

Predicted data

Since the actual data was collected from the real clinic situation where the safety of the patients were most concerned, therefore, the MPA plasma concentrations-time profiles could not be obtained after three different doses for all 23 patients participated in the study. The MPA plasma concentrations, which had not been actually drawn, were predicted for each individual patient (using linear regression equation). Assumption that the relationship between plasma MPA concentrations and MMF doses was linear.

Statistical analysis

Excel 98 (Microsoft) were used for statistics, calculation, and graphic interfaces. The independent samples test in SPSS version 10.0 were used to compare means of the MPA plasma concentrations and MPA AUC between the experienced GAE patients and the none experienced GAE patients. The linear regression analysis in SPSS version 10.0 was used to calculate the correlation between the MPA plasma concentrations and the MPA AUC, leading to the limited sampling strategy.

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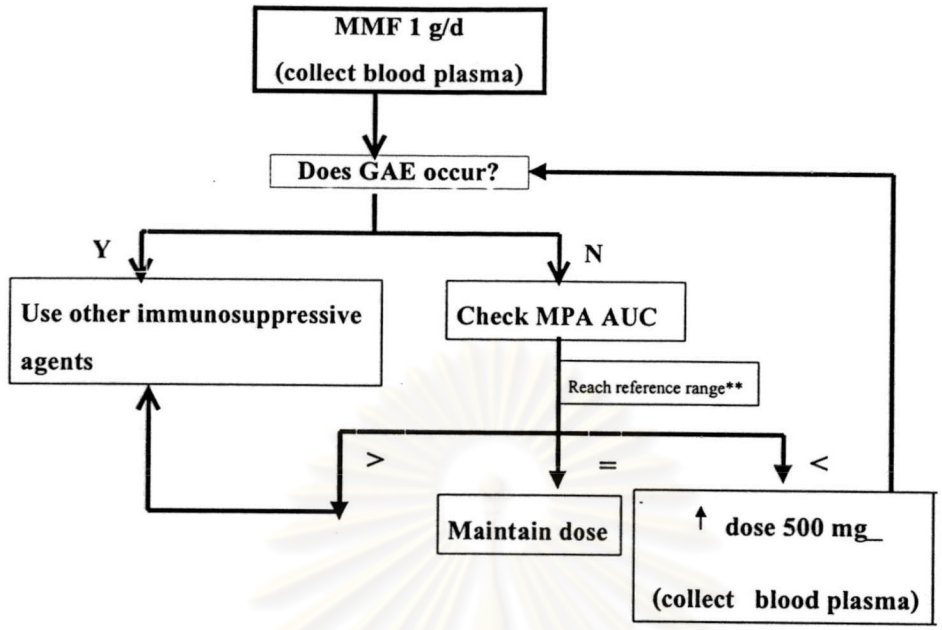
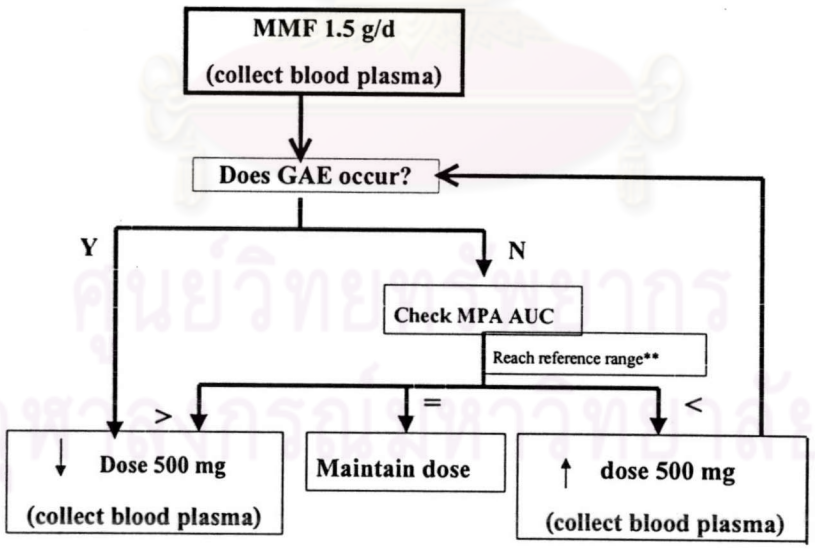


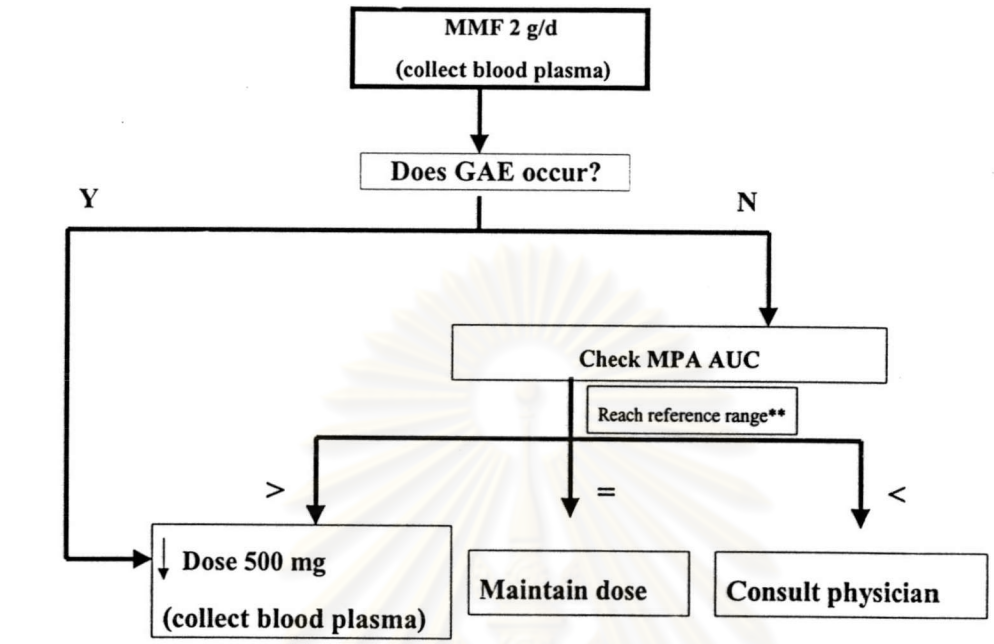
** reference range 30-60 mcg*hr/mL (Shaw LM, et al.)

Figure 1 A : Step of the study for patient who Receiving MMF 1000 mg/d



** reference range 30-60 mcg*hr/mL (Shaw LM, et al.)

Figure 1 B : Step of the study for patient who Receiving MMF 1500 mg/d



** reference range 30-60 mcg*hr/mL (Shaw LM, et al.)

Figure 1 C : Step of the study for patient who Receiving MMF 2000 mg/d

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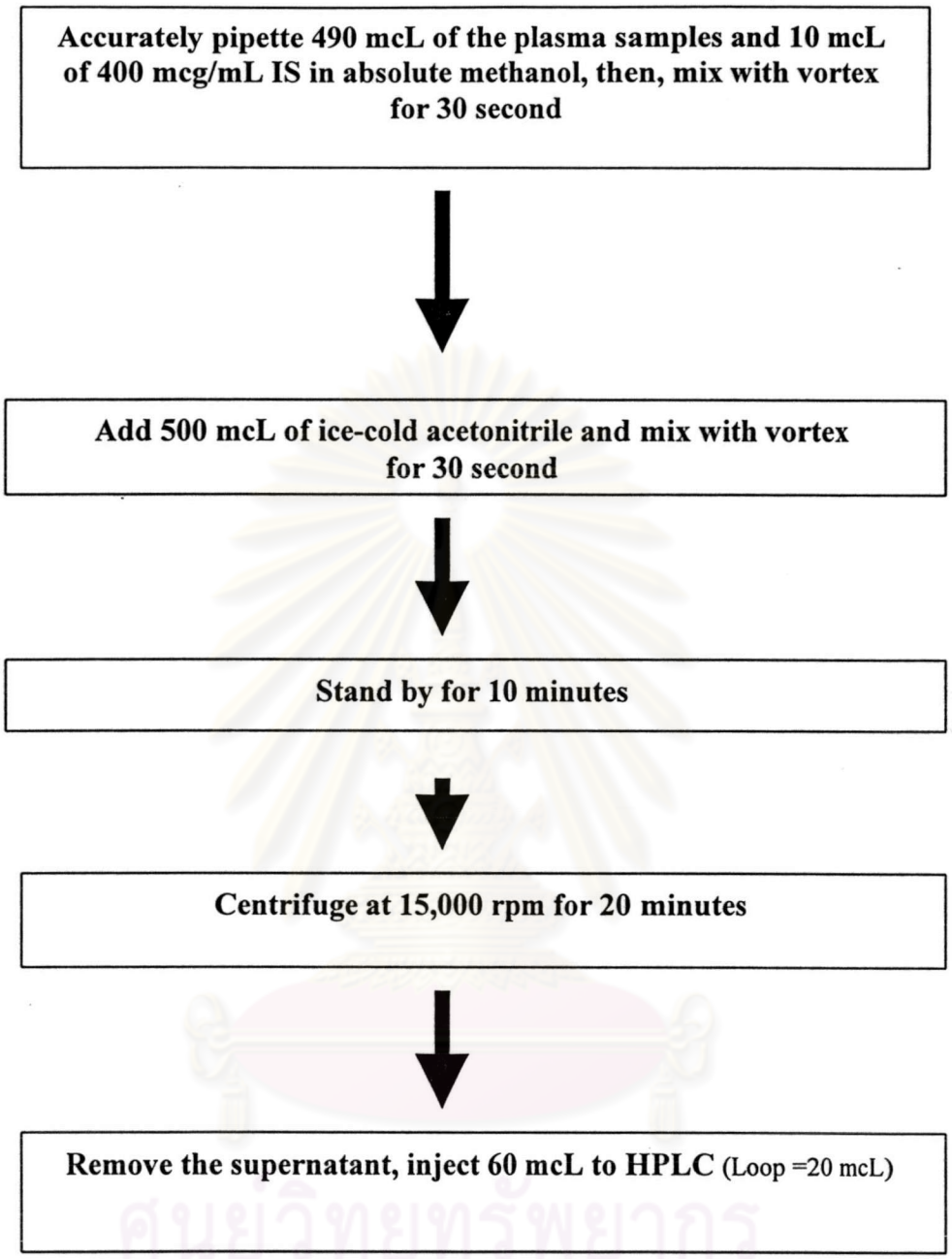

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Figure 2 : Plasma Sample Preparation Method

(modified from the method of Na-Bangchang K, et al.[23] and Hosotsubo H, et al.[24])