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Rapid and simple determination of mycophenolic acid in human plasma by ion-pair RP-LC with fluorescence detection

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Abstract

Mycophenolic acid (MPA) is an immunosuppressive drug given as the prodrug of mycophenolate mofetil (MMF). In order to investigate the pharmacokinetics of MPA, a simple, specific, sensitive and reliable method has been established for the quantitative determination of MPA in plasma from renal transplant recipients. The method involves a single-step protein precipitation procedure and a specific determination by ion-pair reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection. Separation was achieved on a C18 column (150 × 4.6 mm, 5 μm) with a mobile phase composed of borate buffer (pH 10.0; 50 mM) — acetonitrile — tetrabutylammonium bromide (200 mM) (75:25:1, v/v/v). The fluorescence detector was set at 310 (excitation) and 430 nm (emission). Following protein precipitation with ice-cold acetonitrile, clear supernatants (50 μl) were injected into the HPLC system. The retention time of MPA was approximately 4.5 min. The HPLC run time was 8 min. The assay was linear in concentration range 0.2–20.0 μg/ml for MPA in human plasma. Precision of the assay in the concentration range examined was from 0.89 to 3.21% for the intra-assay run and from 3.01 to 4.35% for the inter-assay run. A limit of detection was 0.05 μg/ml at a signal-to-noise ratio of 3. This validated method was then applied to the determination of MPA concentrations in renal transplant recipients after oral administration of 0.75 g of MMF.

Keywords: Mycophenolic acid; Ion-pair reversed-phase HPLC; Fluorescence detection; Pharmacokinetics

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1. Introduction

Mycophenolate mofetil (MMF, CellCept, RS-61443-000), the 2-(4-morpholino) estyl ester of mycophenolic acid (MPA), is an oral prodrug that is rapidly and extensively hydrolyzed by plasma esterases probably in the liver and the intestine to MPA. Furthermore, MPA is primary metabolized by glucuronidation in the liver to MPAG, an inactive metabolite that is normally present in plasma at about 40-fold higher concentrations than MPA. MPA is the active metabolite of MMF, is an antiproliferative immunosuppresant, increasing used after solid-organ transplantation in combination with cyclosporin A (CyA) or tacrolimus (FK-506) [1].

The mechanism of inhibition of the immune response is based on the selective, noncompetitive and reversible inhibition of inosine monophosphate dehydrogenase (IMPDH), the rate controlling enzyme in de novo pathway more than the salvage pathway for purine biosynthesis in T and B cells therefore MPA suppresses lymphocytes more than other neutrophils [2,3]. A depletion of the guanine nucleotide pool in lymphocytes leads to a reduction in DNA synthesis, proliferation of lymphocytes, and subsequent immunosuppression. The role of therapeutic drug monitoring to improve the therapeutic efficacy and to minimize adverse effects is still under investigation.

Some analytical methods have been developed to quantify MPA in biological samples after administration of therapeutic doses. Gas liquid chromatography (GLC) [4], fluorometry [4], and HPLC method with UV detection [5–8] have been developed, but they involve the time-consuming sample pretreatment and are not easily available for routine drug monitoring.

The present study describes a rapid, sensitive and highly selective HPLC method with fluorescence detection, for the determination of MPA in a small volume of plasma sample, with one-step extraction. This procedure could be very useful for carrying out simultaneous studies of the pharmacokinetics and pharmacodynamics of MPA.

2. Experimental

2.1. Reagents and standards

MPA was gift from Roche (Basel, Switzerland). The HPLC-grade acetonitrile, methanol and reagent-grade boric acid, tetrabutylammonium bromide (TBA) were purchased from Wako pure chemical (Osaka, Japan). TBA was dissolved in methanol to make 200 mM/l of solution and stored at room temperature. HPLC-grade water was obtained from Mill-Q water purification system (Millipore, Milford, MA, USA) and throughout the study. MPA was dissolved in methanol to make 1.0 mg/ml of stock solution and stored at −70°C. Quality control samples (0.5, 2.0 and 10.0 μg/ml) and calibration standards were prepared by mixing a drug-free plasma with solutions of MPA. The calibration standards were prepared prior to each assay run. The quality control samples were stored and frozen at −70°C until analysis.

2.2. Instrumentation

The chromatographic system consisted of Shimadzu Model LC-6A pump (Kyoto, Japan) fitted with Rheodyne manual sample injector (Model 7125, Rheodyne) equipped 50 μl sample loop. The detector used was a Shimadzu Model RF-530 fluorescence HPLC monitor. Data were processed with Shimadzu C-R3A chromatograph unit. The separation was performed in Asahipack ODP-50 particle size 5 μm, 6.0 × 150 mm I.D. column (Shodex, Tokyo, Japan), connected a guard column packed with the same bonded phase (6.0 × 10 mm I.D.).

2.3. Chromatographic conditions

The mobile phase used for the ion-pair reversed-phase chromatographic method consisted of a mixture of borate buffer (pH 10.0; 50 mM) — acetonitrile — TBA (200 mM) (75:25:1, v/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 1.0 ml/min. Chromatography was performed

at ambient temperature. Detection was fluorescence, excitation and emission wavelengths were set at 310 and 430 nm, respectively.

2.4. Patients samples

Samples of EDTA plasma were obtained from healthy volunteers or patients with renal transplant recipients treated orally with MMF and centrifuged immediately after venipuncture at $3000 \times g$ for 10 min at a temperature of 4°C. Plasma was transferred to polypropylen tubes and then stored at −70°C until HPLC analysis.

2.5. Sample preparation

The patient samples were stored in freezer at −70°C then allowed to thaw at room temperature before processing. The patient, quality control and standard samples were prepared as follows. A 100 μl volume of plasma was mixed in 1.5 ml Eppendorf tubes with equal volume of ice-cold acetonitrile was then added and the solution was vortex-mixed for 30 s and kept at room temperature for 10–15 min. The mixture was then centrifuged for 10 min at $12\,000 \times g$. A 50-μl aliquot of the clear supernatant was injected into the HPLC system.

2.6. Precision and accuracy

The intra-assay precision and accuracy of the method were evaluated by analyzing on the same day ten replicates of quality control samples against a calibration curve. Inter-assay precision and accuracy were assessed by performing analyses of same quality control samples. The procedure was repeated on different days ($n = 10$).

2.7. Statistical analysis

The statistical analysis was performed using computer program StatView ver 4.5 (Adacus, Concepts, Berkely, CA, USA) on a Macintosh PowerBook 3400 microcomputer.

3. Results and discussion

3.1. Chromatography and detection

MPA demonstrated only minor fluorescence (wavelengths of excitation and emission are 310 and 430 nm, respectively). MPA fluorescence being extremely pH-dependent, only an alkaline mobile phase allowed adequate sensitivity. Bopp and Schirmer [4] had to rely on fluorometric procedure with alkalinization (borate buffer, pH 10.0; 0.5 M) to achieve sufficient sensitivity. Our use of low concentration of borate buffer (pH 10.0; 50 mM) served the apparent pH of the mobile phase to 10 and improving peak shape. Peak shape and separation from endogenous compounds were further optimized by the addition of TBA. The concentration of TBA was optimized in the range from 50 to 500 mM. A concentration of 200 mM proved to be optimal regarding peak shape and separation from endogenous compounds (Fig. 1). An Asahipack ODP-50 column was selected for the solid-phase because it provided a wide pH range from pH 2 to 11.

Fig. 2 shows representative chromatograms obtained in our study. Assays of drug-free plasma showed there were no interfering endogenous peaks, neither was there any interference from other drugs taken by these subjects. The UV

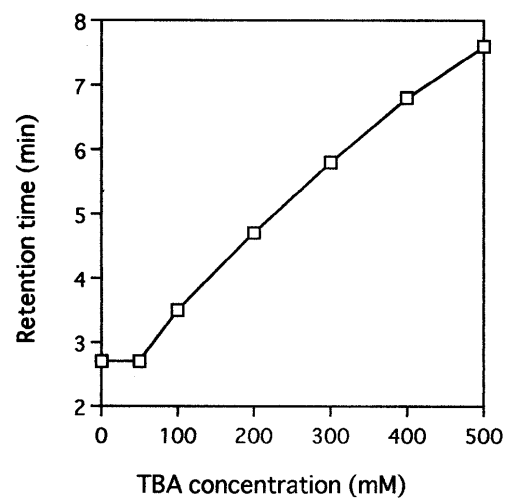

Fig. 1. Effect of pairing ion concentration on the retention time of MPA.

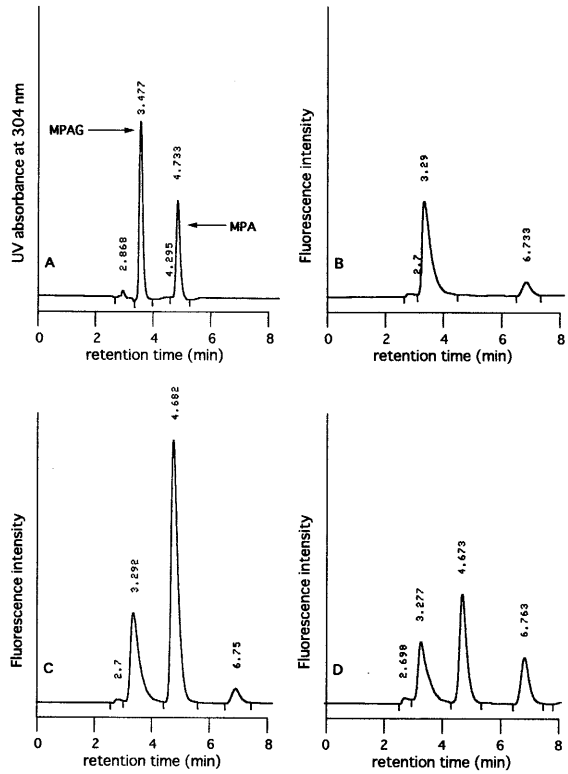

Fig. 2. Typical chromatograms of MPA. Standards of MPAG and MPA in UV detection at 304 nm, (B) blank plasma, (C) spiked plasma sample of 10.0 μg/ml and (D) a patient sample of 4.2 μg/ml.

absorption of MPAG and MPA is same mobile phase was recorded in Fig. 2A. MPAG demonstrated no significant fluorescence property and do not interference at the retention time of MPA. Using this method we did not detect any interference with the MPA peak measured in the plasma samples from more than 200 subjects. The retention time of MPA was 4.7 min, and the overall chromatographic run time was 8 min. Following protein precipitation with acetonitrile, constant volumes (50 μl) of supernatants were injected onto the column. Acetonitrile provided a more efficient precipitation than an equal volume of methanol, and contrarily to phosphoric, did not adversely affect mobile phase pH. We could not find an internal standard with an appropriate retention time and suitable fluorescence properties.

Typically, samples were injected for 12 h. After each day of analysis, the column was rinsed with 50% acetonitrile in water at 1.0 ml/min for 30 min. This ensured good column performance and avoided the accumulation of borate buffer in the system. As a results, retention time and peak area were extremely stable between runs.

3.2. Precision and accuracy

The intra-assay precision for 0.5, 2.0 and 10.0 μg/ml of MPA concentrations was 3.21, 1.78 and 0.89%, respectively. The inter-assay precision and accuracy were studied using the data of same quality controls analyzed over 10 days. The results are shown in Table 1. Precisions expressed as coefficients of variation (C.V.) ranged from 3.01 to 4.35% and accuracy defined as (measured value/theoretical value) × 100 (%) reached approximately 100% for MPA throughout the three concentrations examined.

Table 1
Intra- and inter-assay precision and accuracy results for plasma samples with MPA

Theoretical value (μg/ml)	Measured value (mean ± S.D.) (μg/ml)	Precision C.V. (%)	Accuracy (%)
Intra-assay (n = 10)			
0.5	0.498 ± 0.016	3.21	99.5
2.0	1.912 ± 0.034	1.78	95.6
10.0	10.02 ± 0.089	0.89	100.2
Inter-assay (n = 10)			
0.5	0.495 ± 0.021	4.24	99.0
2.0	2.023 ± 0.088	4.35	101.1
10.0	10.13 ± 0.305	3.01	101.3

Table 2
Absolute recovery of MPA from human plasma

MPA concentration (μg/ml)	Recovery ($n = 10$) mean ± S.D., %)	C.V. (%)
0.5	99.7 ± 4.1	4.11
2.0	99.6 ± 3.6	3.61
10.0	101.1 ± 2.6	2.60

3.3. Linearity

Linearity was tested by analyzing plasma calibration standards containing known (spiked) amounts of MPA at concentrations of 0.2, 0.4, 0.6, 0.8, 1.0, 4.0, 8.0, 12.0, 16.0, and 20.0 μg/ml. The calibration curves were found to be linear in the concentration range 0.2–20.0 μg/ml and passed through the origin on all working days. The mean ± S.D. of the calibration curves yielded the following equations: $y = (1.014 \pm 0.013)x - (0.083 \pm 0.01)$, in which y is the measured concentration and x is the spiked concentration.

3.4. Limit of detection and quantification

The lower limit of detection, with a signal-to-noise ratio of 3, was 0.05 μg/ml for MPA. The sensitivity of the method allowed an easy quantification of 0.1 μg/ml (limit of quantification) of MPA in plasma samples.

3.5. Recovery

The absolute recovery of MPA was assessed at the three 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. Table 2 lists the results of recovery studies.

3.6. Stability of MPA

To test the stability of the samples, spiked plasma sample were left on the stand at room temperature for 24 h before extraction or after extraction, and standard solution was spiked to mobile phase stored at room temperature for 24 h. Results of these studies were compared with the results of spiked samples and standard, which were immediately analyzed. No significant deviation was found from the nominal values. The data in Table 3 show that MPA was stable over a 24 h period at room temperature.

3.7. Specificity and selectivity

Blank plasma from ten different individuals showed no interfering endogenous substances eluting at the retention time of MPA. Potentially coadministered immunosuppressive drugs (CyA, FK-506, steroids, azathiopurine, and mizoribine) were not detected with the described analytical method.

3.8. Application to patient samples

The application of the assay for pharmacokinetic research in renal transplant recipients was demonstrated. The plasma concentration–time profile in patients after oral administration of 0.75 g of MMF as determined by the described analytical methods is shown in Fig. 3.

Table 3
Stability of MPA

Sample	MPA (mean ± S.D., μg/ml) ($N = 5$)
Sample immediately analyzed	3.51 ± 0.08
Sample at room temperature for 24 h before analysis	3.49 ± 0.10
Extract at room temperature for 24 h before analysis	3.53 ± 0.12
Standard immediately analyzed	5.03 ± 0.14
Standard in mobile phase at room temperature for 24 h before analysis	4.96 ± 0.17

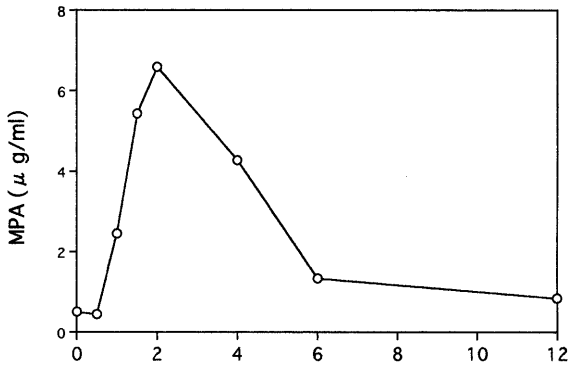

Fig. 3. Plasma concentration–time curve for MPA, after a 0.75-g oral dose of MMF.

4. Conclusion

An analytical method using simple protein precipitation followed by ion-pair reversed-phase high-performance liquid chromatography with fluorescence detection for quantitative determination of MPA in renal transplant recipients has been developed. The present method is, to my knowledge, the first analytical method described for the quantitative determination of MPA with fluorescence detection. The overall performance of HPLC method was found to be satisfactory for the purpose of determining the concentrations of MPA in plasma samples from PK measurements of MPA in renal transplant recipients.

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