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Rapid and simultaneous determination of mycophenolic acid and its glucuronide conjugate in human plasma by ion-pair reversed-phase high-performance liquid chromatography using isocratic elution

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Abstract

A high-performance liquid chromatographic method has been developed for the simultaneous determination of mycophenolic acid (MPA) and its glucuronide conjugate (MPAG) in human plasma. The method involves protein precipitation with acetonitrile, followed by ion-pair reversed-phase chromatography on C_{18} column, with a 40 m*M* tetrabutyl ammonium bromide (TBA)–acetonitrile (65:35, v/v) mobile phase. A 20-μl volume of clear supernatant was injected after centrifugation, and the eluent was monitored at 304 nm. No interference was found either with endogenous substances or with many concurrently used drugs, indicating a good selectivity for the procedure. Calibration curves were linear over a concentration range of 0.5–20.0 μg/ml for MPA and 5–200 μg/ml for MPAG. The accuracy of the method is good, that is, the relative error is below 5%. The intra- and inter-day reproducibility of the analytical method is adequate with relative statistical deviations of 6% or below. The limits of quantification for MPA and MPAG were lower than 0.5 and 5.0 μg/ml, respectively, using 50 μl of plasma. The method was used to determine the pharmacokinetic parameters of MPA and MPAG following oral administration in a patient with renal transplantation.

Keywords: Mycophenolic acid; Mycophenolic acid glucuronide

1. Introduction

Mycophenolate mofetil (RS-61443-000, MMF, Fig. 1), a new immunosuppressive agent, is a morpholinoethyl ester of mycophenolic acid (MPA, Fig. 1), and is used in combination with cyclosporin A (CyA) or tacrolimus (FK506) as an immunosuppressive agent after organ transplantation [1,2]. In vivo, MMF is completely absorbed after oral administration and becomes rapidly converted to its active metabolite (MPA) by plasma esterase. MPA is conjugated to form a phenolic glucuronide conjugate (MPAG, Fig. 1). MPAG is pharmacologically inactive, primarily cleared through the kidney, but due to enterohepatic recirculation it plays an important role

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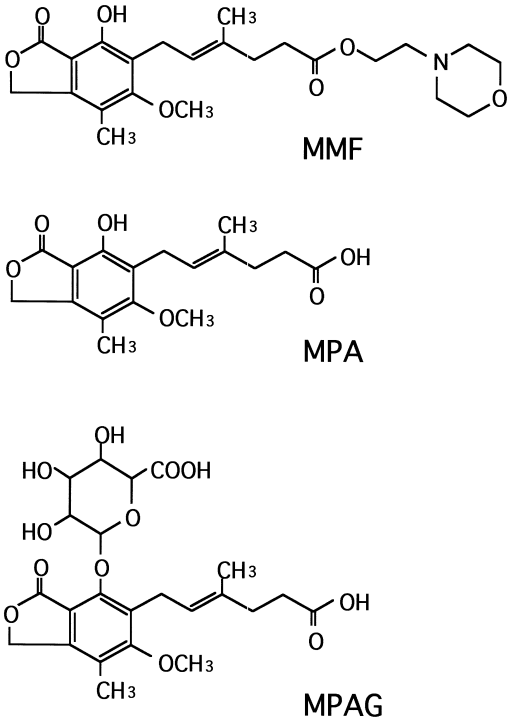

Fig. 1. Chemical structures of mycophenolate mofetil (MMF) and its two main metabolites, mycohenolic acid (MPA) and mycophenolic acid glucuronide conjugate (MPAG).

in the maintenance of steady-state plasma MPA levels [3].

Several high-performance liquid chromatography (HPLC) methods [4–8] have been developed for the determination of MPA and MPAG in plasma. However, they involve the use of gradient elution and time-consuming sample pretreatment and are not very suitable for routine drug monitoring. This reported describes a rapid and selective method for the simultaneous determination of MPA and MPAG in plasma by ion-pair reversed-phase HPLC using isocratic elution.

2. Experimental

2.1. Chemicals

MPA and MPAG were a gift from Roche (Basel, Switzerland). Acetonitrile, methanol (HPLC grade), and tetrabutyl ammonium bromide (TBA; analytical grade) were purchased from Wako (Osaka, Japan). HPLC-grade water was obtained from a Milli-Q water purification system (Millipore, Milford, MA, USA) and used throughout the study.

Standard stock solutions of MPA (1.0 mg/ml) and MPAG (10.0 mg/ml) were individually prepared in methanol, and stored at −70°C. Working solutions were made by dilution with methanol–water (1:1). Standard calibration solutions were prepared by spiking drug-free human plasma with working solution, and then further diluting to achieve final concentrations of between 0.5 to 20.0 μg/ml of MPA, and 5 to 200 μg/ml of MPAG. Quality control samples for assessing precision were prepared in pooled drug-free human plasma at 1.0, 5.0 and 10.0 μg/ml for MPA, and 10, 50 and 100 μg/ml for MPAG. Plasma standards and control samples were dispensed in 0.5-ml aliquots and stored at −70°C.

2.2. Patient samples

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

2.3. Apparatus

The chromatographic system consisted of a Shimadzu Model LC-6A pump (Kyoto, Japan) fitted with a Rheodyne manual sample injector (Model 7125, Rheodyne) equipped with a 20-μl sample loop. The detector used was a Shimadzu Model SPD-6AV with a variable-wavelength UV–Vis detector. Data were processed with a Shimadzu C-R3A chromatograph unit. The separation was performed in a LUNA C_{18} (2) particle size 5 μm, 150×4.6 mm I.D. column (Phenomenex, Torrance, CA, USA), connected to a guard column packed with the same bonded phase (4.0×2.0 mm I.D.).

2.4. Chromatographic conditions

The mobile phase used for the ion-pair reversed-phase chromatographic method consisted of a mixture of 40 m*M* TBA–acetonitrile (65:35, 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 1.2 ml/min. Chromatography was performed at ambient temperature. The analytes were detected at 304 nm, using a setting of 0.01 AUFS.

2.5. Preparation of the sample

The sample for analysis was prepared as follows. A 50-μl volume of plasma and an equal volume of acetonitrile were added to a 1.5-ml Eppendorf tube 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 *g*. A 20-μl aliquot of the clear supernatant was injected into the HPLC system.

2.6. Pharmacokinetic study

One renal transplant recipient was administered an oral dose of 1.5 g/12 h of MMF. Blood samples were drawn before (0, trough) and at various times (0.5, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0 and 12.0 h) after administration.

3. Results

3.1. Chromatography

Typical chromatograms resulting from the analysis of various plasma samples are shown in Fig. 2. MPAG and MPA appear as well resolved peaks with retention times of 3.8 and 6.5 min, respectively. The excellent separation of the MPAG and MPA peaks allowed for quantification by simply measuring the peak area. No chromatographic interferences from endogenous substances or drugs that could be co-administered to renal transplant recipients such as cyclosporin A, tacrolimus, methylpredonisolone, azathioprine, mizoribine, amphotericin B, fluconazole, 5-flurocytosine, vancomycin were found.

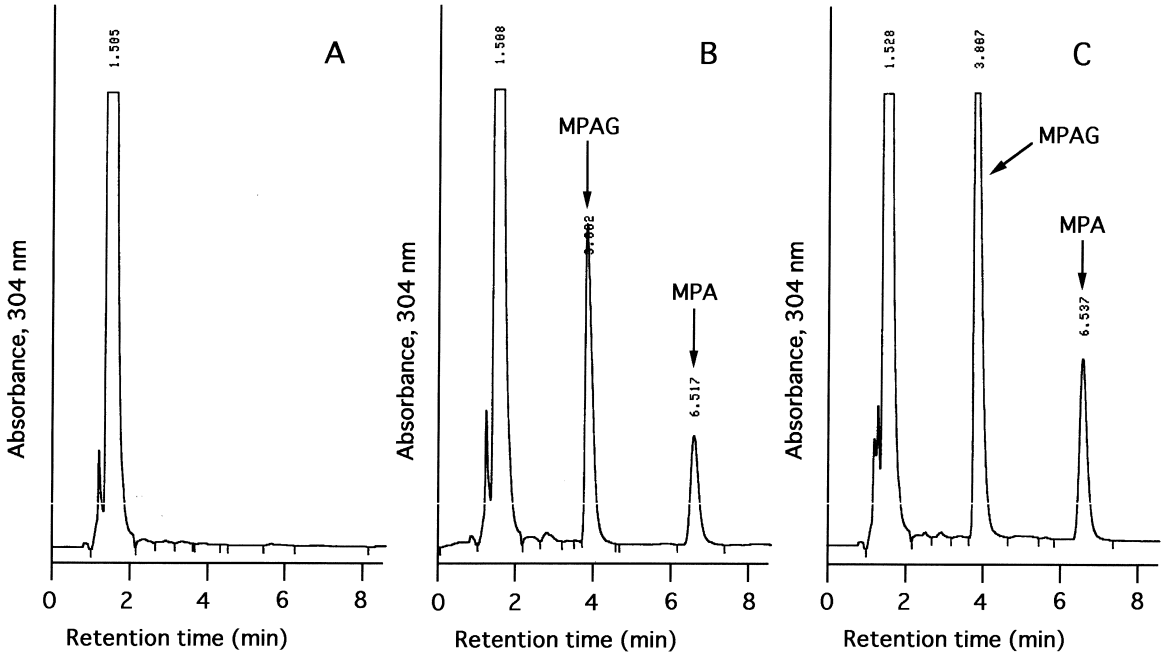

Fig. 2. (A) Chromatogram of a blank plasma sample, (B) plasma sample spiked with MPAG and MPA, and (C) plasma sample taken 1.5 h after the administration of MMF (1.5 g).

Table 1
Accuracy and precision of MPA and MPAG added to plasma

Sample	Added (μg/ml)	Found[a] (μg/ml)	% Error	RSD (%)
MPA	1.0	0.99±0.04	1.0	4.04
	6.0	6.07±0.27	1.7	4.45
	12.0	11.81±0.38	1.6	3.22
	18.0	18.14±0.51	0.8	2.81
MPAG	10	10.3±0.50	3.0	4.85
	60	61.5±2.54	2.5	4.13
	120	118.4±4.22	1.3	3.56
	180	182.7±5.52	1.5	3.02

[a] Mean±SD (n=5).

3.2. Assay validation

An internal standard was found to be unnecessary in this method since the sample preparation was only protein precipitation with acetonitrile. Accuracy and precision data for the described method using spiked MPA and MPAG are shown in Table 1. Intra- and inter-day reproducibility data for MPA and MPAG is shown in Table 2. Relative standard deviations (RSDs) were below 6% for all concentrations for intra-day as well as inter-day precision.

3.3. Recovery

The absolute recoveries of MPA and MPAG calculated by direct comparison of the peak area of the plasma standards with those of the corresponding standards in mobile phase. The mean±SD absolute recovery of MPA and MPAG averaged 98.5±2.4 and 102.4±3.4% (n=5), respectively. They were not statistically different over the range of concentrations studied.

3.4. Linearity

The calibration curves for MPA and MPAG were linear from 0.5 to 20.0 μg/ml (0.5, 1.0, 2.0, 4.0, 8.0, 12.0, 16.0 and 20.0 μg/ml) and 5.0 to 200 μg/ml (5.0, 10, 20, 40, 80, 120, 160 and 200 μg/ml), respectively. The mean±SD (n=5) of their calibration curves yielded the following equations: $y=(0.984\pm0.03)x-(0.01\pm0.02)$ for MPA ($r>0.99$) and $y=(1.03\pm0.03)x+(0.91\pm0.03)$ for MPAG ($r>0.99$), in which y is the measured concentration and x is the spiked concentration.

3.5. Limit of quantification

The limits of detection and quantification were determined according to the method of Car and Waklich [9]. The limit of detection is defined as the amount of analyte giving a peak area three times the maximum noise peak of a blank sample observed at the retention time of each analyte. The limits of detection of the assay were evaluated as 0.1 μg/ml for MPA and 1.0 μg/ml for MPAG. The limits of quantitation for MPA and MPAG were 0.5 and 5.0 μg/ml, respectively.

Table 2
Intra- and inter-day accuracy and precision data

MPA				MPAG			
Added (μg/ml)	Found (μg/ml)	% Error	RSD (%)	Added (μg/ml)	Found (μg/ml)	% Error	RSD (%)
Intra-day							
1.0	1.03±0.05[a]	3.0	4.85	10	10.5±0.45	5.0	4.29
5.0	4.98±0.24	0.4	4.81	50	50.5±1.60	1.0	3.17
10.0	9.77±0.34	2.3	3.48	100	98.2±2.05	1.8	2.09
Inter-day							
1.0	1.04±0.06[b]	4.0	5.77	10	9.7±0.49	3.0	5.05
5.0	5.04±0.27	0.8	5.36	50	48.7±2.18	2.6	4.47
10.0	10.15±0.40	0.4	3.94	100	101.8±4.03	1.8	3.96

[a] Mean±SD (n=10).
[b] Mean±SD (n=5).

Table 3
Stability of MPA and MPAG

Sample	MPA (μg/ml)	MPAG (μg/ml)
Sample immediately analysed	3.64±0.16[a]	74.5±2.4
Sample at room temperature for 24 h before analysis	3.70±1.5	73.8±2.3
Extract at room temperature for 24 h before analysis	3.62±1.5	74.6±2.1
Standard immediately analysed	4.99±0.21	50.4±2.01
Standard in mobile phase at room temperature for 24 h before analysis	5.01±0.22	50.2±1.83

[a] Mean±SD (n=5).

3.6. Stability of MPA and MPAG

To test the stability of the samples, spiked plasma samples were left on the stand at room temperature (25°C) for 24 h before extraction or after extraction, and standard solution was spiked to mobile phase stored at room temperature. Results of these studies were compared with the results of spiked samples and standard which were immediately analysed. No significant deviation was found from the nominal values. The data in Table 3 show that MPA and MPAG were stable over a 24-h period at room temperature.

3.7. Clinical application

The present method has been applied to the pharmacokinetic study of MPA and MPAG. One renal transplant recipient receiving oral MMF (1.5 g) twice daily was investigated, and Fig. 3 shows an example of the plasma concentration–time curve of MPA and MPAG. Following oral administration of MMF, the highest concentrations of MPA and MPAG were observed at 2.0 h and 6.0 h, respectively, being 14.8 μg/ml and 171.3 μg/ml, respectively. The area under the curve ($AUC_{0-12\ h}$) was 77.5 μg/ml/h for MPA and 1674 μg/ml/h for MPAG.

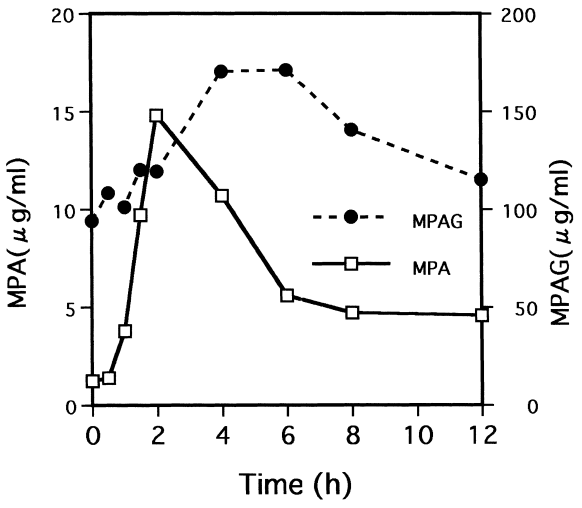

Fig. 3. Plasma concentration–time curve for MPA and MPAG, after a 1.5 g oral dose of MMF.

4. Discussion

Most published methods for quantifying MPA and MPAG on plasma samples used solid-phase [5] or liquid–liquid extraction [6], and sometimes evaporation of the organic extract under a nitrogen stream [8]. With our isocratic ion-pair HPLC method, we could easily and completely separate the MPA and MPAG of interest from potentially interfering material. In contrast, multi-step extraction and gradient elution were work intensive and time-consuming [4]. The sample pretreatment procedure, involving a direct deproteinisation with acetonitrile, is simple and rapid, avoiding degradation of the drug. Tsina et al. reported solid-phase extraction of aliquots of 0.5 ml of plasma using C_{18} cartridges [4].

We developed the new HPLC mobile phase using TBA as the ion-pair reagent to bind the carboxylic acid group of MPA and MPAG. The use of a TBA ion-pair reagent gave a sufficient retention time, which was faster than that in the study of Huang et al. [7]. A variety of mobile phase compositions were evaluated, and the selection of the final mobile phase and the concentration of the ion-pair reagent was based on baseline separation of the MPA and en-

hanced retention for MPAG from peaks of endogenous impurities.

Most HPLC methods involve UV absorbance at 215 [6,8] or 254 nm [4,5,7], in order to increase the sensitivity of the assay. However, under such conditions, there is more risk of interference by other co-administered drugs or endogenous substances. The specificity of the method for MPA and MPAG is also increased by using a detection wavelength of 304 nm as opposed to 215 or 254 nm as many possible interfering peaks from the biological matrix are not seen at this wavelength. A detection wavelength of 304 nm was therefore suitable for the quantification of MPA and MPAG.

5. Conclusion

An analytical method using simple extraction followed by ion-pair reversed-phase HPLC for quantitative determination of MPA and MPAG in human plasma has been developed. The method is suitable for determination of the drug plasma levels in patients undergoing clinical investigations and will prove useful for evaluating the bioavailability and pharmacokinetic/pharmacodynamic properties of these novel immunosuppressive drugs.

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