

DETERMINATION OF MYCOPHENOLIC ACID IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A simple, sensitive and reproducible HPLC method is presented for determination of mycophenolic acid (MPA) in human plasma. Samples were prepared after precipitation of the plasma protein by addition of acetonitrile and naproxen was used as internal standard (I.S.). Separation was performed by reversed-phase HPLC, using a Hamilton PRP-C18 Column, 51% acetonitrile and 49% potassium phosphate buffer (20 mM) at pH 3.0 as mobile phase, flow rate of 1.0 ml/min, and UV detection at 215 nm. MPA and I.S. had retention times of 7.5 and 11.35 min, respectively. The method showed an acceptable linearity in the range of 0.1 µg/ml-40 µg/ml with r^2 of .9992. The concentration of 0.1 µg/ml was determined as quantification limit. Mean absolute recovery was 94.8%. The mean intra- and inter-day reproducibility of method was 4.6 and 11.4% respectively.

Key Words: Mycophenolic acid, Mycophenolate mofetil, HPLC, Plasma, Kidney transplant

INTRODUCTION

Mycophenolate mofetil (MMF) the morpholinoethylester of mycophenolic acid (MPA) (Fig 1) is an immunosuppressive agent which its efficacy in prevention of rejection in transplant recipients has been proved. MMF may be considered as a prodrug, since its immunosuppressive activity is expressed only after hydrolysis to MPA (1).

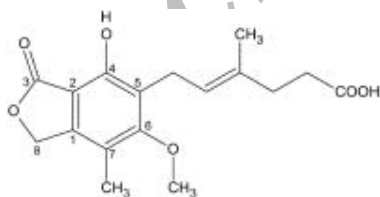


Figure 1. Mycophenolic acid structure: (4E)-6-(4-hydroxy - 6-methoxy -7 -methyl - 3- oxo - 1,3- dihydro-2-benzofuran-5-yl)-4-methylhex-4-enoic acid.

MPA is a potent, selective, uncompetitive and reversible inhibitor of inosine monophosphate dehydrogenase, and as a result inhibits the de novo pathway of guanosine nucleotide synthesis without incorporation into DNA. Unlike most other cells, lymphocytes rely on the de novo pathway more than the salvage pathway (hypoxanthine guanine

phosphoribosyl transferase, HGPRT) for purine biosynthesis (2, 3).

An oral dose of MMF is hydrolyzed rapidly during first-pass metabolism to MPA which is further metabolized to mycophenolic acid glucuronide (MPAG) (4).

MPAG is the main metabolite of MPA and pharmacologically inactive. Up to 87% of MPAG is excreted in the urine and remainder is secreted into the bile and is subjected to enterohepatic circulation. In the intestine, deglucuronidation of MPAG to MPA takes place which is then reabsorbed and results in a second MPA concentration maximum in plasma 6-12h after the initial intake of MMF. At clinically relevant concentrations, 97% of MPA and 82% of MPAG are bound to plasma albumin, and over 99.9% of MPA has an extracellular plasma distribution. For this reason, plasma is the preferred medium for measurement of the concentration of MPA (2).

MMF is administered at a fixed dose, but unlike other immunosuppressants its active form is not routinely monitored (5). Recent investigations have shown high interindividual variability in the pharmacokinetic parameters of MPA, suggesting a benefit of monitoring MPA concentration in kidney and heart recipients. (6).

Several methods for quantification of MMF, MPA and the MPAG have been reported. These include

bioassay (7-9), TLC (10), GC (11, 12) and HPLC (1-6, 13-36).

Of the HPLC methods which have been developed to measure the concentration of MPA in plasma, some methods measure MPA alone (13, 14, 16-20, 25), some methods measure MPA and its phenol- and acylglucuronide metabolites (1), some methods measure MPA and MMF (23, 26) and in the other methods MPA and MPAG are measured (2, 3, 4, 15, 21, 22, 24, 27, 28). However time-consuming and laborious extraction (3, 4, 13-22), large volume of sample required (23), gradient HPLC procedure (2, 14, 24-26), use of ion pairing agent (13, 27, 28), and running at high temperature (1) are their disadvantages.

In this article a rapid and sensitive reversed phase HPLC method for the routine determination of MPA in plasma is described.

MATERIAL AND METHODS

Materials

Mycophenolic acid was obtained from Biocon (Bangalore, India). Naproxen was a gift from Ruzdaru (Tehran, Iran). Acetonitrile, methanol (HPLC grade), potassium dihydrogenphosphate and phosphoric acid (analytical grade) were purchased from Merck (Darmstadt, Germany). Laboratory-prepared distilled water was used throughout the experiments. Plasma was prepared from healthy volunteers.

Preparation of standard solutions

A stock solution containing 1mg/ml of MPA was prepared in methanol and stored at -20°C . The stock solution was further diluted by methanol/water (50/50) to obtain working solution of 200 $\mu\text{g/ml}$.

The internal standard (naproxen) was prepared as a stock solution of 1mg/ml in methanol. A working I.S. solution was prepared by dilution of stock solution in methanol.

Apparatus and chromatographic conditions

A Knauer 1001 HPLC pump and a Knauer 2600 UV detector was used. For instrumental control, data collection and processing Chromgate software version 2.55 was employed. (Dr. Ing. Herbert Knauer GmbH Scientific Instrument Germany)

Chromatographic separation was performed using a Hamilton PRP-1 reversed phase column (250mm \times 4.6mm i.d., particle size 10 μm) (Hamilton company, Reno, Nevada), connected to a suitable Hamilton guard column (25 \times 2.3 mm i.d., particle

size 12-20 μm) (Hamilton company, Reno, Nevada). The mobile phase consisted of acetonitrile:0.02M potassium dihydrogen phosphate buffer (pH was adjusted to 3 by addition of 85% phosphoric acid) (51:49 v/v) and was pumped at flow rate of 1ml/min. The column maintained at ambient temperature and the UV detector was set at 215nm. Samples were introduced to HPLC column through a Rheodyne injector fitted with a 50 μl loop.

Sample preparation

To 100 μl of plasma blank or plasma MPA standard was added 10 μl of 40 $\mu\text{g/ml}$ naproxen as internal standard and then 100 μl of acetonitrile. The mixture was vortex mixed for 1min and centrifuged at 10000g for 10min. Fifty μl of clear supernatant was then injected on to the HPLC column.

Preparation of calibration standards

During method validation, eleven point calibration standards were prepared in plasma for each run, covering a concentration range of 0.1- 40 $\mu\text{g/ml}$. Calibration data were acquired by plotting the ratio of MPA peak areas to I.S. peak areas against the concentration of the calibration standards, followed by a linear-regression analyses.

Selectivity and specificity

Control human plasma which was obtained from three healthy subjects, was assessed by the procedure as described above and compared with the respective spiked plasma samples to evaluate selectivity of the method. Cyclosporin, prednisolone and diltiazem (common coadministrative drugs) were also assessed for potential interferences.

Accuracy, precision, limit of quantification (LOQ) and recovery

Accuracy was determined by replicate analysis of four different levels (0.25, 1, 12, 40 $\mu\text{g/ml}$) and comparing the differences between spiked and those values which were found. Intra-day precision of the assay was studied by triplicate analysis ($n = 3$) of samples at each of six spiked concentrations within the same day. Inter-day precision of the method was determined at the same six concentration which were used in the study of intra-day precision, samples at each concentration was analyzed in triplicates over three consecutive days. The limit of quantification was determined as the lowest point on the

calibration curve that could be analyzed within 20% of the nominal value (absolute percentage errors within 20%). The absolute recovery of MPA was assessed by triplicate analyses of five spiked plasma concentrations in comparison with aqueous concentrations.

Application of the method

A series of blood samples (pre-dose, 0.33, 0.66, 1, 2, 3, 4, 6, 7, 8, 9, 10, and 12h) were collected over a 12-h period after administration of 1g MMF to a 37 year-old, male renal transplant recipient which voluntarily participated in the study and signed an informed consent form. The venous blood samples were collected into vacutainer tubes containing EDTA. Samples were centrifuged (10min, 10,000 rpm), plasma were removed and stored at -70 °C until analysis.

RESULTS

Selectivity and chromatography

Chromatograms of samples of drug-free plasma, spiked plasma and plasma which were obtained from a recipient of kidney transplantation are shown in figure 2. The MPA and I. S. were resolved with the retention times of 7.5min and 11.35min, respectively.

Chromatograms of three healthy subject's plasma did not show any interference at the retention times of MPA and I.S. None of the drug mentioned above interfered with the analyte peaks.

Linearity

Eleven point calibration curves for MPA on separate days were linear over the concentration range of 0.1-40 µg/ml. The mean regression line showed the correlation coefficient (r^2) of 0.9992 with the equation of $y = 0.1403x + 0.0002$ (Table 1).

Table 1. Calibration curve characteristics

	Mean	SD	CV (%)
Slope	0.1403	0.0035	2.5
Intercept	0.0002	5.8×10^{-5}	29
r^2	0.9992	0.0068	0.6

Limit of quantification

The limit of quantification as previously defined was 0.1 µg/ml.

Recovery, accuracy and precision

Absolute analytical recoveries of the assay in five concentrations are shown in table 2. Mean absolute recovery was 94.8%.

The method proved to be accurate and precise; the accuracy at four concentration levels ranged from 91.6-109% (Table 3). The results of within-day and between-days reproducibility are shown in table 4. The within-day and between-days precision ranged from 2.1-7.8% and 1.2-19.3%, respectively.

Application of the method

To demonstrate the usefulness of the proposed method for real analyses of plasma samples, plasma specimens which were taken at different times from a patient under MMF therapy were processed and analyzed and results which are shown in figure 3, demonstrate the potential and soundness of the described approach. The time profile has the typical shape expected for the particular administration scheme of MPA.

Table 2. Absolute analytical recovery of the assay (n=3)

MPA Conc. (µg/ml)	Recovery (Mean ± SD)	CV (%)
1	86±1.4	1.6
4	91±1.5	1.6
12	104±1.5	1.4
24	102±2.6	2.6
40	91±1.5	1.7

DISCUSSION

Several HPLC methods have been developed to measure the concentration of MPA in plasma. The early reported method (23) for determination of MPA in biological fluids requires a 5ml sample volume to determine the plasma MPA concentration and to resolve MPA and its esters and requires a very unusual portion of tetrahydrofuran (15%) and heptansulfonic acid (40%) as mobile phase. A selective and sensitive isocratic HPLC assay using a relatively small volume of the sample (29) for determination of MMF, MPA and MPAG in rat plasma, bile and tissue homogenate and human plasma was reported later but the detection limit for all compounds was 0.17µg/ml. Following the above preliminary methods many other methods have been reported. In some methods gradient HPLC procedure (2, 14, 24-26) and column switching (16) or dual detector system (30) have been employed which are not used in routine analytical procedures. Solid phase extraction (3, 4, 10, 17-21) and liquid-liquid extraction (31) have also been used for sample treatment but they are time consuming and some are expensive. Direct protein precipitation has also

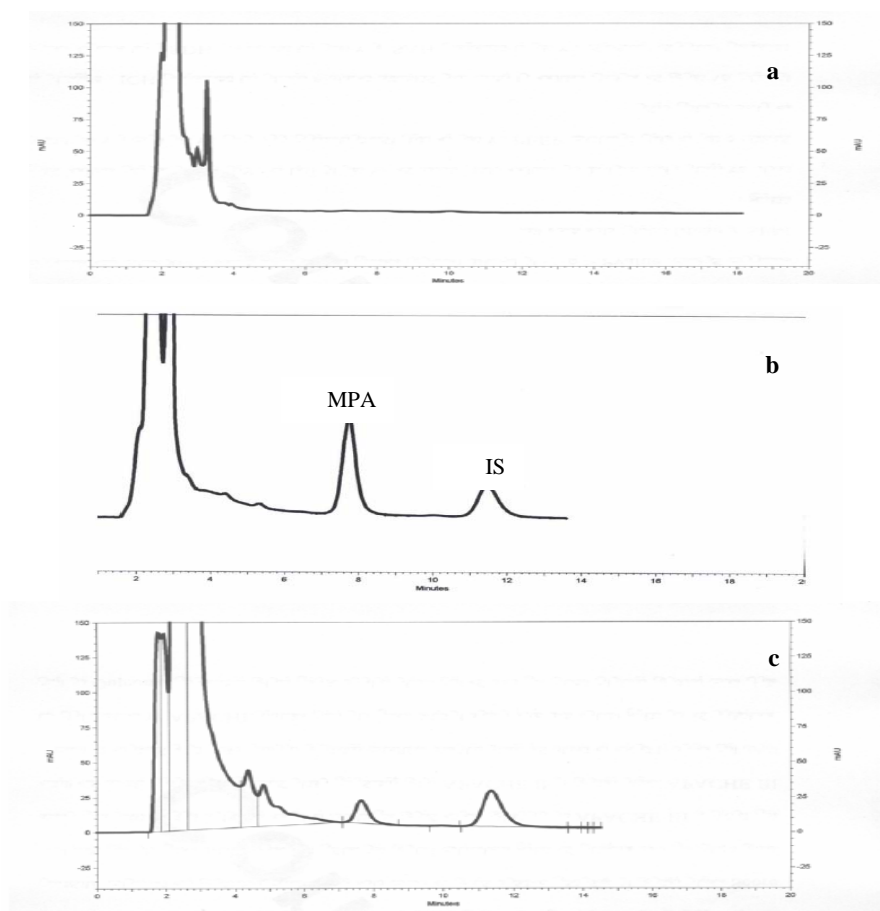


Figure 2. Chromatograms of extract of (a): drug-free plasma, (b): spiked plasma with MPA(16 µg/ml) and IS (2µg/ml), (c): plasma obtained from a male recipient of kidney transplantation at 20 min after an oral dose of 1g MMF. (Retention times of MPA and I.S. were 7.5 and 11.35 min, respectively).

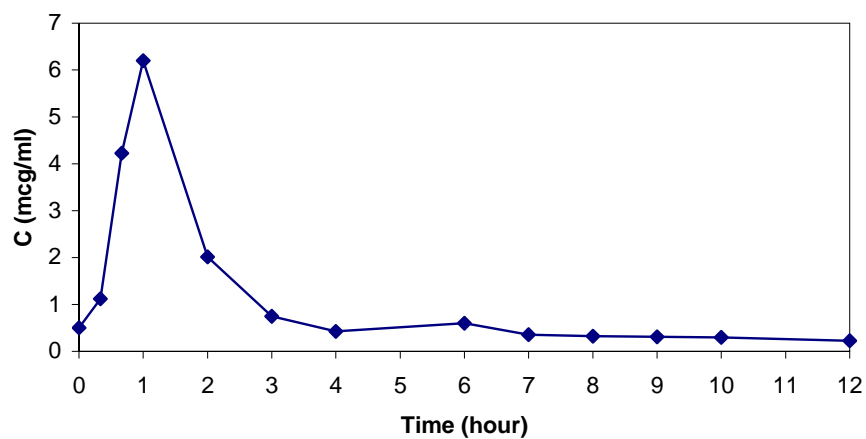


Figure. 3. Plasma concentration-time profile of a male recipient of kidney transplantation following an oral dose of 1g MMF in steady state.

Table 3. Accuracy of the MPA assay

	Drug concentration ($\mu\text{g/ml}$)	Accuracy (%) (Mean \pm SD)	CV (%)	%Error
Within-day	0.25	108 \pm 4.7	4.3	8.1
	1	91 \pm 1.5	1.6	8.3
	12	104 \pm 1.5	1.4	4.9
	40	98 \pm 1.6	1.7	1.3
Between-day	0.25	107 \pm 11	10.5	7
	1	102 \pm 8.9	8.7	2
	12	109 \pm 4	3.8	9.2
	40	99 \pm 1.2	1.2	0.5

Table 4. Within – day and between-day (for 3 days) precision obtained on drug free plasma samples spiked with variable amount of MPA (n=3)

	MPA Conc. ($\mu\text{g/ml}$)	SD	CV (%)
Within-day	0.1	0.0006	3.8
	0.25	0.003	7.6
	0.4	0.004	7.8
	0.5	0.005	6
	8	0.025	2.5
	40	0.10	2.1
Between-day	0.1	0.003	19.3
	0.25	0.04	10.4
	0.4	0.009	17.3
	0.5	0.074	13.7
	8	0.074	6.5
	40	0.07	1.2

been used for sample preparation (1, 13, 15, 25, 27, 28, 30, 32-36). In one method (32) a temperature of 80 °C has been applied, which may cause a possible drug degradation (according to the manufacture, MPA powder should be stored under 4 °C). In another method, an evaporation and reconstitution following direct protein precipitation has been described (33). Hosotsubo et al (28) have also used a direct protein precipitation, but the high limit of quantification of 0.5 $\mu\text{g/ml}$ limits the usefulness of this method. The method reported by Khoshsorur and Erva also uses a direct protein precipitation, but the column temperature of 50 °C limits the usefulness of this method (1). Ion pair containing mobile phases which has been applied in some previously reported methods (13, 27 and 28) shorten the column life. Although in our method MPAG could

not be determined (MPAG is pharmacologically inactive and in routine clinical monitoring of MMF it is not monitored), but this method has some advantages over previously reported methods as it uses a simple sample treatment, a small sample volume, an ambient temperature, and a non sophisticated HPLC system.

CONCLUSION

The simplicity of HPLC conditions, the small sample volume (100 μl), rapid sample preparation and high sensitivity, makes our method applicable to routine monitoring of MPA in renal transplant recipients.

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