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High-performance liquid chromatographic method for the determination of mycophenolate mofetil in human plasma

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Abstract

A method for the quantification of mycophenolate mofetil (MMF, CellCept) in plasma using solid-phase extraction and HPLC is described here. A solution of internal standard is added to a 0.5-ml plasma aliquot. The resulting sample is treated with water and dilute HCl and applied to a C_{18} solid-phase extraction column. After a water wash, the MMF and internal standard are eluted with methanol–0.1 *M* citrate–phosphate buffer, pH 2.6 (80:20, v/v). A 20-μl aliquot of the eluate is injected onto a C_{18} column (5 μm particle size, 150×4.6 mm I.D.) and eluted at ambient temperature with acetonitrile–0.05 *M* citrate–phosphate buffer, pH 3.6, containing 0.02 *M* heptanesulfonic acid (41:59, v/v). Quantification is achieved by UV detection at 254 nm. The method is reproducible, accurate and specific for MMF. Using 0.5 ml of plasma for analysis, the quantification limit is 0.400 μg/ml and the range is 0.400–20 μg/ml. Based on the stability profile of MMF in plasma, it is recommended that blood samples collected following intravenous infusion be immediately stored on ice and that plasma be prepared rapidly, immediately stored frozen at −80°C and analyzed within four months of collection.

Keywords: Mycophenolate mofetil

1. Introduction

Mycophenolate mofetil (MMF, CellCept, RS-61443-000, I, Fig. 1), the 2-(4-morpholino)ethyl ester of mycophenolic acid (MPA, II, Fig. 1), has been shown in double-blind, randomized, controlled clinical trials to be effective in adjunctive therapy with cyclosporine and corticosteroids for the prevention of acute rejection in patients receiving kidney transplants [1–3]. It has been approved as an immunosuppressive agent for prevention of rejection following kidney transplantation and is being evaluated for other indications.

Following oral administration of MMF to animals, MMF is not detected in the plasma; after bolus intravenous administration of MMF to animals, only a trace amount is detected in the plasma [4,5]. MMF is hydrolyzed to form free MPA, which is the active metabolite [6]. MPA is conjugated to form a phenolic glucuronide conjugate (MPAG, III, Fig. 1) [6], which is pharmacologically inactive but may be hydrolyzed in vivo to form free MPA. MPA potently, selectively and reversibly inhibits inosine monophosphate dehydrogenase (IMPDH) and therefore inhibits the de

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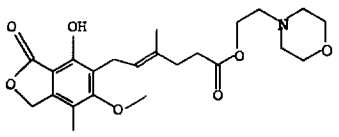
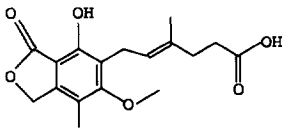
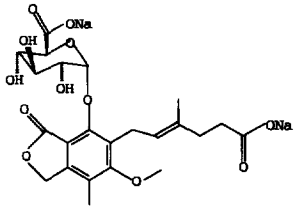


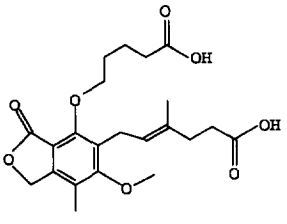
Mycophenolate Mofetil
RS-61443
Structure I



Mycophenolic Acid (MPA)
Structure II



Mycophenolic Acid Glucuronide
(MPAG)
Structure III



Internal Standard
RS-60461-000
Structure IV

Fig. 1. Structures of mycophenolate mofetil, its metabolites and the internal standard.

novo pathway of purine synthesis in T and B cells (T and B lymphocytes) [7,8]. Unlike most other cells, lymphocytes rely on the de novo pathway more than the salvage pathway (hypoxanthine-guanine phosphoribosyl transferase; HGPRT) for purine biosynthesis.

MMF is potentially present in the plasma during and immediately following intravenous (i.v.) infusion. Therefore, in addition to HPLC methods for determination of MPA and MPAG [9], an HPLC method was developed for the determination of MMF in plasma and is described here. Although the details of an HPLC method for determination of MMF in rat tissue homogenates were reported recently [5], that method differs from the one reported here in many respects.

2. Experimental

2.1. Chemicals and supplies

Mycophenolate mofetil, 2-(4-morpholino)ethyl-(*E*) - 6 - (1,3 - dihydro - 4 - hydroxy - 6 - methoxy - 7 - methyl - 3 - oxo - 5 - isobenzofuranyl) - 4 - methyl - 4 - hexenoate (I; Fig. 1) and internal standard, RS-60461-000, (*E*) - 6 - [1,3 - dihydro - 4 - (4 - carboxybutoxy) - 6 - methoxy - 7 - methyl - 3 - oxo - 5 -

isobenzofuranyl] - 4 - methyl - 4 - hexenoic acid (I.S., i.v.; Fig. 1), were obtained from Syntex Research (Palo Alto, CA, USA). HPLC-grade methanol and acetonitrile were purchased from Burdick and Jackson Laboratories (Muskegon, MI, USA) and water was purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA). Analytical-grade sodium phosphate dibasic anhydrous, analytical-grade citric acid monohydrate and 0.1 *M* hydrochloric acid were purchased from Mallinckrodt (St. Louis, MO, USA). Heptanesulfonic acid was purchased from Eastman Kodak (Rochester, NY, USA). Heparinized human control plasma was obtained from normal, healthy volunteers from the Clinical Studies Unit, Syntex Research. Solid-phase extraction columns (Bond Elut LRC) C_{18}, 3 ml, containing 200 mg of sorbent, were purchased from Varian Sample Preparation Products (Harbor City, CA, USA). Aqueous solutions of 0.1 *M* sodium phosphate dibasic, 0.1 *M* citric acid, 0.1 *M* citrate–phosphate buffer (pH 3.6) and 0.05 *M* citrate–phosphate buffer (pH 3.6), were prepared in house.

2.2. Instrumentation

The HPLC system consisted of a Hewlett-Packard Model 1090 liquid chromatograph (Hewlett-Packard, Santa Clara, CA, USA) equipped with an autosampler and a ternary solvent delivery system, a Kratos Spectroflow 785A variable-wavelength detector (Kratos Analytical Instruments, Ramsey, NJ, USA) and a Nelson 6000 Laboratory Data System (PE-Nelson, Cupertino, CA, USA).

2.3. Chromatographic conditions

A 20-μl aliquot of sample extract was injected onto an Adsorbosphere HS C_{18}, 5-μm, 150×4.6 mm I.D. column (Alltech Associates, Deerfield, IL, USA) at ambient temperature using a mobile phase of acetonitrile–0.05 *M* citrate–phosphate buffer (pH 3.6), containing 0.02 *M* heptanesulfonic acid (41:59, v/v). The UV detector was set at 254 nm and the flow-rate was 0.4 ml/min. A C_{18} guard column (Keystone Scientific, Bellefonte, PA, USA) and a 0.5-μm precolumn filter (Upchurch Scientific, Oak Harbor, WA, USA) were connected to the analytical column and were replaced after every 200–500 injections.

2.4. Sample preparation

2.4.1. Spiking procedure

A stock solution of MMF (100 μg/ml) in acetonitrile was prepared and further diluted with acetonitrile to prepare spiking solutions at concentrations of 2, 3, 4 and 10 μg/ml. The MMF solutions were stored in a refrigerator no longer than 2 months. A stock solution of the internal standard (200 μg/ml) in methanol was prepared and further diluted in methanol–water (9:1) to prepare an internal standard spiking solution with a concentration of 10 μg/ml. For preparation of the calibration standards used for construction of the calibration curve and for validation of the method, an appropriate volume (0.1 or 0.2 ml) of one of the MMF spiking solutions or the stock MMF solution was added to 0.5-ml aliquots of blank human plasma. This procedure was used to prepare calibration standards at MMF concentrations of 0.400, 0.600, 0.800, 1.20, 4.00 and 20.0 μg/ml.

2.4.2. Extraction of calibration standards

To each calibration standard, 1.5 ml of water, 0.1 ml of the internal standard spiking solution and 0.75 ml of 0.1 *M* HCl were added. Each sample was mixed in a vortex-mixer for 10 s and applied to a C_{18} solid-phase extraction column that had been preconditioned with 2 ml of methanol followed by 2 ml of water using gravity flow and then allowed to drip dry. The plasma mixture was allowed to pass through the column under the force of gravity. The test tube that had contained the plasma was washed with 1 ml of water, and the wash was applied to the same solid-phase extraction column. The column was allowed to drip dry and the eluate was discarded. The column was then eluted with methanol–0.1 *M* citrate–phosphate buffer, pH 2.6 (80:20, v/v), and the eluate was collected in an HPLC autosampler vial. The eluate was mixed briefly and an aliquot of 20 μl was injected onto the HPLC system for analysis.

2.4.3. Extraction of clinical samples

Samples of the heparinized plasma obtained from healthy volunteers or from patients treated intraven-

ously with MMF were stored at −80°C prior to analysis. Samples were thawed for approximately 15 min in a 25°C water bath, mixed in a vortex-mixer for 30 s and centrifuged for 2 min at approximately 110–200 *g*. An aliquot of 0.5 ml was then used for analysis. The samples were extracted using the procedure described for the calibration standards. The concentration of MMF in the samples was calculated by reference to calibration curves generated from calibration standards analyzed along with each batch of clinical samples.

2.5. Data handling and calculations

Linear least-squares regression was performed on the peak-height ratio (analyte peak height/internal standard peak height) versus concentration data generated by the calibration standards to construct a linear standard curve of the form

peak-height ratio=m(concentration)+b .

Calibration standards with MMF concentrations of 0.400 to 4.00 μg/ml were used in the unweighted linear regression to construct a calibration curve. A calibration standard of 20 μg/ml was used to verify extrapolations of the curve up to this MMF concentration. Concentrations in unknowns were then determined from their peak-height ratios by the standard curve equation with appropriate corrections for sample aliquot volumes less than 0.500 ml.

For determination of validation parameters, MMF calibration standards at each point of the standard curve (in addition to those used for curve generation; 0.400, 0.600, 0.800, 1.20, 2.00, 4.00 and 20.0 μg/ml) and MMF quality control samples (see below; 0.794, 3.96 and 18.3 μg/ml) were prepared and analyzed. Four replicates of each standard or control were analyzed within the same run to determine intra-assay parameters. One sample of each standard or control was analyzed in each of four (for standards) or eight (for controls) runs to determine inter-assay parameters.

2.6. Preparation of quality control samples

Quality control (QC) samples prepared by spiking MMF into control human plasma were stored at −80°C in a manner similar to that used for the clinical samples. QC samples were prepared at the following three MMF concentrations: QC 1 (0.794 μg/ml), QC 2 (3.96 μg/ml) and QC 3 (18.3 μg/ml). To prepare the bulk QC 1, a 40 μg/ml spiking solution was prepared from the 100 μg/ml stock MMF solution, and the appropriate amount was added to 50 ml of control human plasma. To prepare the bulk QC 2, the appropriate volume of the 100 μg/ml stock MMF solution was evaporated to dryness and redissolved in 0.5 ml of methanol. Control human plasma was added to the resulting MMF solution. To prepare the bulk QC 3, the appropriate amount of MMF was weighed, sonicated with 0.5 ml of methanol and mixed in a vortex-mixer to dissolve the MMF. Control human plasma was added to the MMF solution. The three bulk QC plasma samples were swirled briefly and stirred on a magnetic stirrer for 5 min before being apportioned into polypropylene tubes for storage at −80°C. Two QC samples at each of the three different concentrations were analyzed with each batch of clinical samples to monitor the assay performance and possible degradation of MMF in the QC samples stored under the same conditions as the study samples.

3. Results and discussion

3.1. Quantification limits

The quantification limit of the method is 0.400 μg/ml using 0.5 ml of plasma for analysis. Concentrations below 0.400 μg/ml are reported as below the quantification limit (BQL). The quantification limit was set as the lowest MMF concentration at which the signal-to-noise ratio of the HPLC peak for MMF was at least 8:1 and also good intra- and inter-assay coefficient of variation (C.V.) values (≤10%) and recoveries (90–110%) for calibration standards were achieved. At an MMF concentration of 0.400 μg/ml, the signal-to-noise ratio of the HPLC peak for MMF was approximately 8:1. For the 0.400 μg/ml calibration standard, the intra- and inter-assay C.V. values were 1.84 and 4.46% and the intra- and inter-assay mean recoveries were 105 and 101%, respectively.

3.2. Precision and accuracy

The precision of the method was assessed by the intra-assay (within day) and inter-assay (between day) C.V. values. The accuracy of the method was evaluated by recovery, defined as the ratio of the concentration of MMF found to that added (found/added, expressed as a percentage). Data for the intra- and inter-assay C.V. values and for recoveries obtained using calibration standards are presented in Table 1. Similar data is also presented for the QC samples in Table 1. All C.V. values were less than 5% for the calibration standards and less than 4% for the QC samples. All recoveries were between 90% and 105% for both calibration standards and QC samples.

3.3. Specificity

The analysis of blank human plasma from six different sources showed no interfering peaks at the retention times of MMF and the internal standard. Typical chromatograms obtained from the analysis of blank human plasma are presented in Fig. 2a. A chromatogram obtained from the analysis of human plasma spiked with an MMF concentration of 0.400 μg/ml is presented in Fig. 2b. The presence of MPA or MPAG in the plasma sample does not interfere with the assay. Fig. 2c shows a chromatogram for the analysis of MMF in the sample collected from a patient during i.v. infusion of MMF.

3.4. Linearity

The linear calibrated MMF concentration range of the method is 0.400 to 4.00 μg/ml, using 0.5 ml of plasma for analysis. Correlation coefficients were generally $\geq$0.999. As demonstrated by the data in Table 1, the good recoveries for quality controls (90.7–105%) confirmed the accuracy of the method. The range of the method can be extrapolated to 20.0 μg/ml by verification with a 20.0 μg/ml standard, as described above. The good recoveries for the 20.0 μg/ml standard analyzed in each run confirmed the accuracy of the extrapolation to 20.0 μg/ml.

3.5. Absolute recovery

The absolute recovery of MMF from plasma, as determined by the analysis of plasma spiked with [^{14}C]MMF, was 90%.

3.6. Stability

MMF in human blood and plasma was found to show temperature-dependent degradation to produce MPA and other products that were not identified. At room temperature (20–23°C), 90% of MMF remained in whole blood after 2.0 h; at refrigerator temperature (1–4°C), 90% remained in whole blood after 7.6 h. In plasma, 90% remained after 3.2 h at 20–23°C and 90% remained after 6.8 h at 1–4°C. No loss of MMF was detected in extracts of plasma stored on the autosampler for two days at ambient

Table 1
Intra- and inter-assay precision and accuracy data for MMF

	Nominal concentration (μg/ml)	n		Mean concentration found (μg/ml)		C.V. (%)		Mean recovery (%)	
		Intra-assay	Inter-assay	Intra-assay	Inter-assay	Intra-assay	Inter-assay	Intra-assay	Intra-assay
Calibration standards	0.400	4	4	0.420	0.402	1.84	4.46	105	101
	0.600	4	4	0.612	0.622	1.05	4.86	102	104
	0.800	4	4	0.790	0.794	0.478	2.13	98.8	99.5
	1.20	4	4	1.19	1.20	0.482	1.32	99.2	100
	2.00	4	4	1.97	1.98	0.941	0.778	98.5	99.0
	4.00	4	4	3.98	4.00	0.819	0.500	99.5	100
	20.0	4	4	21.0	19.9	1.20	1.95	105	99.5
QC samples									
QC 1	0.794	4	8	0.832	0.819	4.09	2.80	105	103
QC 2	3.96	4	8	4.16	4.00	2.24	3.35	105	101
QC 3	18.3	4	8	17.1	16.6	1.26	2.75	93.4	90.7

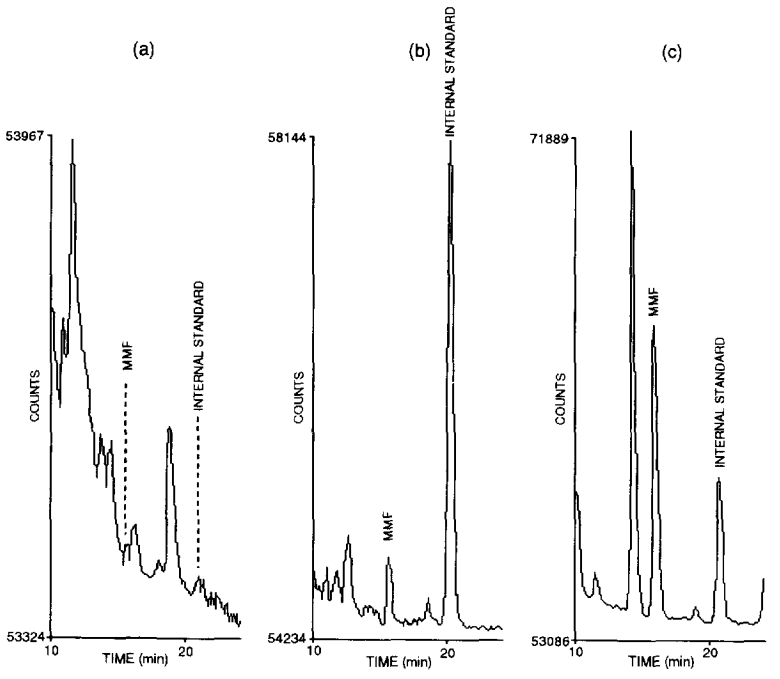


Fig. 2. Representative chromatograms obtained from the analysis of MMF in (a) blank human control plasma, (b) blank human control plasma spiked with 0.400 μg/ml of MMF and (c) plasma sample collected from a patient during i.v. infusion of MMF.

temperature, stored on the lab bench (20–23°C) for seven days or stored in a refrigerator (0–4°C) for two weeks prior to analysis.

MMF in QC samples was stable at −80°C for four months, at which time testing ceased. MMF in QC samples slowly degraded upon storage at −20°C; only 70%–75% of the initial MMF concentrations in QC samples remained after 39 days at that temperature. In plasma at −20°C, the time required for the MMF concentration to fall to 90% of its initial value (T_{90}) was approximately six days, and the estimated half-life (T_{50}) for MMF in plasma at −20°C was 86 days. However, good stability was seen with storage at −80°C, as stated above.

Concentrations of MMF in human plasma were relatively unaffected by cycles of freeze and thaw. Less than 11% loss of MMF in QC samples was seen after the samples were subjected to two cycles of freeze and thaw, with freezing at −80°C.

3.7. Application

The method reported here for determination of MPA in human plasma differs in many major respects from the method reported by Sugioka et al. [5] for determination of MPA in rat tissue homogenates. Besides the different sample matrix, Sugioka et al. used different sample processing procedures (protein precipitation with methanol, followed by liquid–liquid extraction), different HPLC conditions [Shim-pack CLC-CN cyano column, elevated (50°C) column temperature and a detection wavelength of 304 nm] and a different method of standard curve construction (peak-area ratios). Although the quantification limit of the method for tissue homogenates is reported as 1.1 μg/ml, no statistical parameters at the limit of quantification were provided. Nevertheless, it is likely that the sample processing procedures and HPLC conditions in the method reported here contribute to the lack of chromatographic interferences at 254 nm and enable a quantification limit of 0.400 μg/ml with good precision and accuracy.

The method for plasma reported here has been applied to the analysis of plasma from transplant patients treated with MMF by i.v. infusion. A representative profile from one such patient is shown in Fig. 3. The MPA and MPAG profiles for that

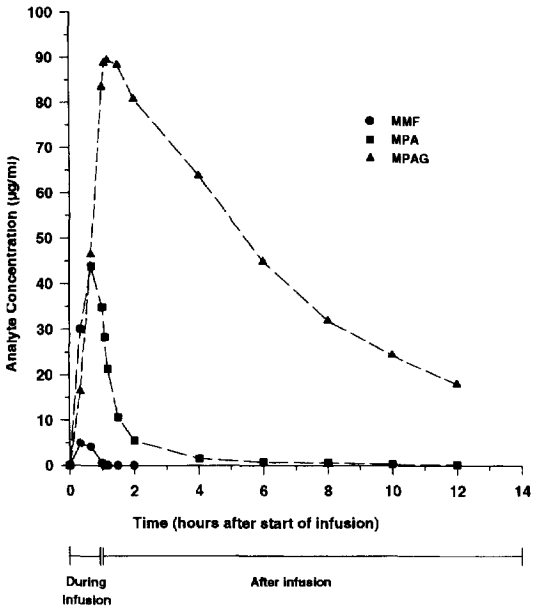


Fig. 3. Plasma concentration versus time profiles of MMF, MPA and MPAG from a transplant patient following intravenous infusion of 1750 mg of MMF given in 60 min. Values reported as BQL are plotted as 0 μg/ml.

patient are also shown in Fig. 3. The MPA concentrations represented in Fig. 3 are total MPA concentrations determined by HPLC [9]; the potential utility of determining the free fraction of MPA in plasma from subjects with specific physiological conditions has been suggested by others [10].

4. Conclusions

The HPLC method described here for the determination of MMF in human plasma is precise, accurate and specific for MMF. MMF is stable during the analytical procedure; however, specific precautions are recommended to minimize the slow degradation that occurs during blood sample collection and processing to produce plasma. These precautions include immediate storage on ice, rapid processing, and frozen storage of plasma at −80°C. Analysis within four months is recommended.

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