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Technical note

Rapid determination of mycophenolic acid in plasma by reversed-phase high-performance liquid chromatography

Anna Pastore[a,*], Anna Lo Russo[a], Fiorella Piemonte[b], Liliana Mannucci[a], Giorgio Federici[a]

[a]*Laboratory of Clinical Biochemistry, Children's Hospital and Research Institute "Bambino Gesù", Viale Oxford, 81 00133 Rome, Italy*

[b]*Molecular Medicine Unit, Children's Hospital and Research Institute "Bambino Gesù", Piazza S. Onofrio, 4-00165 Rome, Italy*

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Abstract

A simple, accurate and sensitive high-performance liquid chromatographic method with UV detection was carried out to measure plasma concentrations of mycophenolic acid. Following a simplified acid hydrolysis of the sample, the separation was carried out in 4 min using a Zorbax® Eclipse® C_8 reversed-phase column with a flow-rate of 1.5 ml/min, and monitoring the absorbance at 250 nm. Throughput was up to 100 samples in 24 h. Within the investigated concentration ranges of mycophenolic acid (0–100 mg/l), good linearity ($r>0.99$) was obtained. The method is sensitive (the limit of detection was about 20 μg/l) and precise (for 0.49 mg/l added to plasma, within-run C.V. was 2% and between-run was 4.2%; for 2.88 mg/l, within-run C.V. was 0.35% and between-run C.V. was 0.69%; for 24.38 mg/l, within-run C.V. was 0.77% and between-run C.V. was 3.1%). Analytical recoveries were 96% for 0.5 mg/l mycophenolic acid added to plasma, 100% for 12 mg/l and 102.5% for 24 mg/l.

Keywords: Mycophenolic acid

1. Introduction

Mycophenolic acid (MPA), the active metabolite of mycophenolate mofetil (MMF), was originally developed as a potential antibiotic, antineoplastic and antipsoriatic drug; its immunosuppressant properties and use in transplantation were established only recently [1–3]. MMF targets the de novo purine biosynthesis pathway by noncompetitive, selective and reversible inhibition of inosine monophosphate dehydrogenase; this leads to an arrest of proliferation and function of T and B lymphocytes [4,5]. MMF is a prodrug formulated to enhance active MPA bioavailability [6]; it is rapidly hydrolyzed after oral administration and peak MPA concentrations occur within the first hour after dosing [7]. MPA is then converted mainly to an inactive glucuronide metabolite (MPAG), which is eliminated by urinary excretion [8].

Several HPLC methods had been developed to measure the concentration of MPA in plasma [9–13], but they were time-consuming or use laborious extraction protocols.

*Corresponding author. Tel./fax: +39-6-2090-2270.
E-mail address: apastore@opbg.net (A. Pastore).

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Here we described a quick and sensitive reversed-phase HPLC method for the determination of MPA in plasma after a simplified acid treatment of the sample. The simple sample preparation, the remarkably low sample volumes required for the assay and the good reproducibility represent an advance of this method over the other already published methods.

2. Experimental

2.1. Chemicals

All chemicals were of analytical grade and were readily available commercial products.

2.2. Equipment

The HPLC-system was an Agilent Technologies 1100 Series equipped with the BinPump G1312A, the DEGASSER G1322A, the auto sampler ALS G1313A and the detector DAD G1315B operating at a wavelength of 250 nm. All the operation, such as the injection cycle, was controlled by the ChemStation® program; the data obtained were analyzed with the ChemStation® program (Agilent Technologies Deutschland GmbH, Waldbronn, Germany).

2.3. Preparation of standards and quality controls

A standard stock solution containing 10 mg/l MPA was made in ethanol and stored at −20 °C. Ten standards ranging from 0.39 to 100 mg/l were prepared diluting the stock solution in drug-free plasma and stored in 100-μl aliquots at −80 °C. Quality controls were prepared from a separate stock solution of MPA (10 mg/l). Nominal concentrations of 0.5, 12 and 24 mg/l in drug-free plasma were used to assess low-, medium- and high-quality controls, respectively.

2.4. Sample preparation and HPLC analysis

Samples were prepared as follows: 100 μl of plasma or standards or controls were spiked with 50 μl of sulfosalicylic acid (100 ml/l). The solutions were vortex mixed for 60 s and centrifuged at 1000 *g*

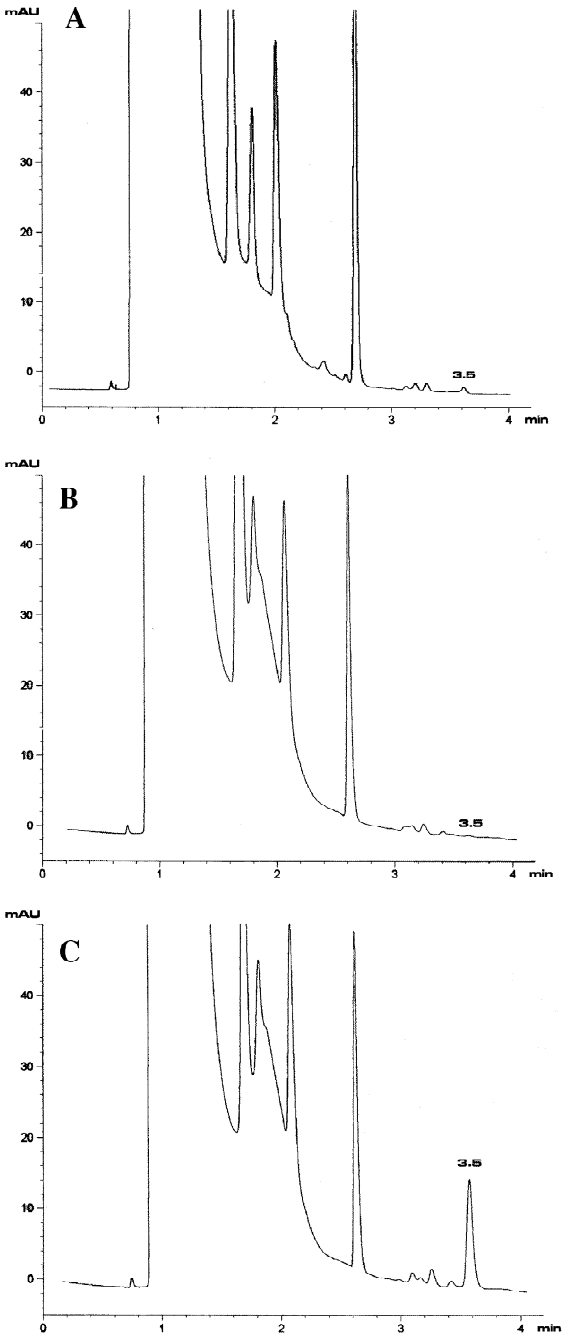

Fig. 1. Chromatograms of: (A) human plasma sample containing an MPA concentration near the lower limit of quantification. The peak corresponds to an MPA concentration of 0.1 mg/l; (B) human plasma sample obtained from a renal transplant patient not on MMF therapy; (C) human plasma of a subject after administration of known dose of MPA. The peak corresponds to an MPA concentration of 2.88 mg/l. MPA retention time was 3.5 min.

for 5 min. Fifty μl of sample were then injected into a Zorbax® Eclipse® XDB-C_8 column (150×4.6 mm; 5 μm particle size; Agilent Technologies) equilibrated with a solution containing 20 m*M* KH_2PO_4 in acetonitrile (250 ml/l), pH 3 (A). MPA was eluted from the column with a retention time of 3.5 min at ambient temperature with a gradient of acetonitrile (B) (0 min, 10% B; 1–4 min, linear gradient to 35% B), at a flow-rate of 1.5 ml/min. Total HPLC run time was 7 min; re-equilibration time was 2 min. Stability of MPA in the precipitation reagent is 24 h at room temperature.

3. Results and discussion

Under the conditions used, the eluted peak of MPA was distinctly separated. Unidentified peaks appeared which did not interfere with the peaks of interest. The retention time of MPA was 3.5 min. Fig. 1A shows the chromatogram of a human plasma near the lower limit of quantification of MPA. Fig. 1B show the chromatogram of a plasma sample obtained from a renal transplant patient not on MMF therapy; Fig. 1C shows the chromatogram of a plasma sample of a subject after intravenous administration of a known dose of MMF.

In Table 1A the assay precision results are reported. The intra-assay precision was obtained by analyzing 10 replicates of the biological samples on the same day, with the process starting at the extraction step. The inter-assay precision was determined by analyzing the same biological samples on 10 different days over 1 month. For the recovery study, known concentrations of MPA were added to the plasma sample; the concentrations in biological samples were determined in five replicates, with the process starting at the extraction step, and analytical recoveries were calculated (Table 1B). Calibration curves (0.0975–100 mg/l MPA, $n=10$) were prepared in duplicate, with the duplication process starting at the extraction step, by diluting the stock solution in a pooled plasma. A linear relation was obtained between MPA peak area and the expected compound concentration. Correlation coefficients were >0.99, and the equation for the regression line ($n=10$) was: $y = 0.057x + 0.019$ (where y is the peak area and x is the concentration of the analyte). The standard error of the slope was 0.03; the intercept was not significantly different from zero. The limit of detection, defined as the concentration that produces a signal-to-noise ratio of 3, was about 20 μg/l.

Potential chromatographic interference by commonly administered drugs was evaluated by analysis of patient specimens received for routine drug monitoring, including transplant patients under immunosuppressive therapy without MMF and drug-containing methanol standards. In addition, the existence of endogenous chromatographic interferences was evaluated by separate analysis of 40 patient specimens, including transplant recipients without MMF

Table 1
(A) Imprecision of the assay; (B) analytical recovery of the assay

(A)	Intra-assay ($n=10$)			Inter-assay ($n=10$)		
	Mean (mg/l)	SD	C.V. (%)	Mean (mg/l)	SD	C.V. (%)
Mycophenolic acid	0.49	0.01	2.0	0.47	0.02	4.2
	2.88	0.01	0.35	2.88	0.02	0.69
	24.38	0.19	0.77	24.32	0.76	3.1

(B)	Added (mg/l)	Measured[a] (mg/l)	Mean recovered (%)
Mycophenolic acid (added to drug-free plasma)	0.5	0.48 (0.04)	96
	12	12.03 (0.47)	100
	24	24.60 (0.20)	102.5

[a] Mean of five replicate values; SDs are indicated in parentheses.

Table 2
Drugs found not to interfere with the assay

Amikacin	Carbamazepine
Cyclosporine A	Digoxin
Ethosuximide	Phenobarbital
Phenytoin	Salicylate
Tacrolimus	Vancomycin

therapy, sent to the laboratory for routine clinical chemical tests. Table 2 reports the drugs tested to not interfere with the MPA assay.

In conclusion, this HPLC assay for plasma is sensitive and precise and provides a high sample throughput. In addition, the method is quick and simple, and required low sample volumes, providing a reliable determination of MPA in human plasma, allowing thus a precise determination of MPA in pediatric patients receiving MMF therapy.

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