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Short communication

Simple method for the quantitation of mycophenolic acid in human plasma

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Abstract

A simple and rapid isocratic reversed-phase high-performance liquid chromatographic method using UV detection was developed for the quantitation of mycophenolic acid (MPA) in human plasma. The assay was sufficiently robust to allow the analysis of up to 100 samples in a single analytical run. © 1997 Elsevier Science B.V.

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1. Introduction

Mycophenolic acid was first isolated from the fungus *Penicillium brevicompactum* [1]. In the 1960s and 1970s it was investigated as an anticancer agent and for the treatment of rheumatoid arthritis and psoriasis [2]. More recently it has been shown to prolong allograft survival in animal models and has been used effectively to prevent rejection in patients receiving kidney transplants [3,4].

The immunosuppressive effect of MPA results from its potent, selective and reversible inhibition of inosine monophosphate dehydrogenase (IMPDH) inhibiting the de novo pathway of purine synthesis in lymphocytes [1–7]. Lymphocytes rely on the de novo pathway more than the salvage pathway for purine biosynthesis therefore MPA suppresses lymphocytes more than other neutrophils [2,6,7].

Methods for the determination of MPA in plasma using HPLC have extracted MPA from plasma components using solid-phase extraction [1,3,6] or by precipitation of proteins [8–10]. Solid-phase extraction generally results in excellent absolute recoveries of MPA from plasma, however, endogenous plasma components can also be strongly retained ultimately resulting in frequent changes of precolumn. Extraction of MPA from plasma by precipitation can result in poor recovery of MPA [10].

2. Experimental

2.1. Chemicals

Acetonitrile and methanol were HPLC grade from Mallinckrodt Baker (Paris, KY, USA). Phosphoric acid and perchloric acid were analar grade from Fisons (Poole, UK) and BDH (Loughborough, UK) respectively. Mycophenolic acid and naproxen internal standard were obtained from Sigma–Aldrich (Castle Hill, Australia).

Plasma from five separate donors was obtained from Canterbury Health Laboratories (Christchurch, New Zealand).

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2.2. Apparatus

Kontron Autosampler 460, Kontron HPLC pumps 420 and a UVIKON 735LC UV detector were used. Data was collected and analyzed by the Kontron AT Data System 450 vers 3.01.

2.3. Chromatographic conditions

The mobile phase consisted of acetonitrile–0.05% H_3PO_4 (30:70, v/v) and was filtered through a 0.45-μm Millipore apparatus. The column used was an Applied Biosystems Brownlee column 100×4.6 mm RP-18, 5 μm with a 15×3.2 mm Brownlee Newguard precolumn RP-18, 7-μm cartridge. The UV detector was set at 254 nm and the flow-rate was 1.5 ml/min.

2.4. Standards

MPA and naproxen primary standards were prepared by accurately weighing between 2 and 5 mg of compound into a 10-ml volumetric flask and making up to volume with methanol. Primary standards were stored at 5°C and used to prepare fresh aqueous working standards of naproxen (30 μg/ml) and MPA (200 μg/ml) for each analytical run. The MPA working standard was used to prepare fresh plasma standards for each analytical run in the following concentrations: 20, 10, 5, 2.5, 1.0, 0.5, 0.25 and 0.1 μg/ml by the addition of 1000, 500, 250, 125, 50, 12.5 and 5 μl of MPA working standard to labeled 10 ml volumetric flasks and making up to volume with drug free plasma.

Bulk MPA plasma standards for freeze–thaw (FT) stability determinations were prepared in single 20 ml aliquots in the following concentrations: 18.0, 4.0 and 0.4 μg/ml. MPA plasma quality control standards were prepared in the same concentrations as the bulk standards and stored in multiple 4-ml aliquots for assay with each analytical run. Both plasma bulk standards and quality controls were stored at −35°C until assayed. Quality control standards were discarded once thawed and assayed.

2.5. Sample preparation

To 0.5 ml of plasma blank or plasma MPA standard was added 100 μl of 30 μg/ml naproxen internal standard. The mixture was vortexed briefly and proteins denatured by heating in a water bath at 80°C for 5 min. Upon removal from that water bath 25 μl of 1 *M* $HClO_4$ was added followed by 875 μl of methanol. The mixture was vortexed for 30 s and then centrifuged at 3000 *g* for 30 min, 700 μl of clear supernatant was eluted into a clear chromacol small volume vial and sealed. An 80-μl volume was injected onto the column.

2.6. Stability

Freeze–thaw stability was determined by thawing, assaying and then refreezing the bulk MPA standards over four consecutive days. The stability of MPA at concentrations of 20 and 0.25 μg/ml and naproxen internal standard in plasma precipitate at 20°C was determined by hourly measurement of MPA and naproxen peak heights over 24 h.

2.7. Precision and accuracy

The precision of the assay was assessed by the intra- and inter-assay C.V. values. Accuracy was defined as the ratio of the concentration of MPA found to the known concentration expressed as a percentage.

2.8. Extraction efficiency

The absolute recoveries of MPA over the range 0.1–20 μg/ml and naproxen from plasma were determined by peak height comparisons of MPA and naproxen standards from the validation plasma standard curve ($n=4$ separate chromatographic determinations for each concentration in the validation standard curve) with duplicate standards of the same concentration prepared in H_2O–CH_3OH (2:3, v/v).

2.9. Limit of detection

The limit of detection for this assay was defined as the smallest concentration of MPA with an inter-day C.V.>20% ($n=4$) and a mean inter-day accuracy of (100±20)%.

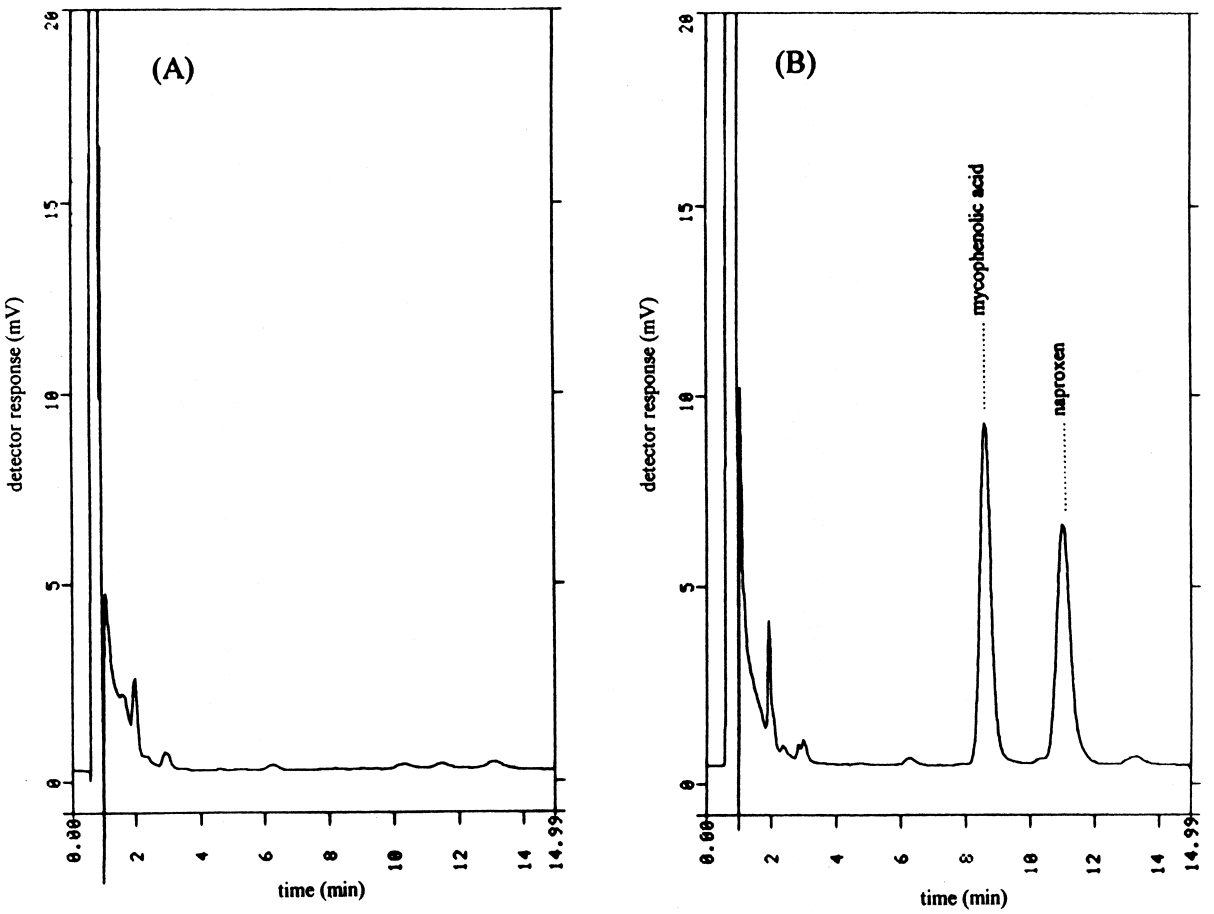

Fig. 1. Chromatograms of (A) blank plasma and (B) plasma spiked with 10 μg/ml MPA and naproxen internal standard.

3. Results

3.1. Chromatography

Shown in Fig. 1 are typical chromatograms of (A) blank plasma and (B) plasma spiked with 10 μg/ml MPA and the internal standard naproxen. MPA and the naproxen eluted at 8.8 and 11.2 min respectively. More than 500 samples were injected onto the column before a change of precolumn was required.

3.2. Stability

MPA was found to be stable for at least four freeze–thaw cycles when stored at −35°C (see Table 1). Both MPA and naproxen standards in methanol

Table 1
Freeze–thaw (FT) stability data over four freeze–thaw cycles for MPA bulk standard stored at −35°C

Bulk standard concentration (μg/ml)	Mean concentration found (n=4 FT determinations)	C.V. (%)	Accuracy (%)
18.0	18.11	0.85	100.6
4.0	4.01	2.45	100.3
0.4	0.42	7.1	105.0

Table 2
Intra- and inter-assay precision and accuracy for MPA

Concentration (μg/ml)	Mean concentrations found (μg/ml)		C.V. (%)		Accuracy (%)	
	Intra (n=4)[a]	Inter (n=8)[b]	Intra (n=4)[a]	Inter (n=8)[b]	Intra (n=4)[a]	Inter (n=8)[b]
20	20.05	20.0	3.56	0.16	100.3	100.0
10	9.94	10.01	2.02	0.58	99.4	100.1
5	4.91	4.98	1.27	0.87	98.2	99.6
2.5	2.51	2.52	2.45	1.41	100.4	100.8
1.0	1.05	1.04	1.96	1.76	105.0	104.0
0.5	0.51	0.48	1.96	3.73	102.0	96.0
0.25	0.25	0.24	8.0	4.06	100.0	96.0
0.1	0.09	0.10	11.1	10.49	90.0	100.0
Q controls						
QC 18.0	18.26	18.19	1.42	1.03	101.4	101.6
QC 4.0	4.04	4.06	0.86	0.84	101.0	101.5
QC 0.4	0.40	0.39	6.46	5.13	100.0	97.5

[a] Data from the validation standard curve (n=4 chromatographic determinations per standard curve concentration in a single run).
[b] Data from eight separate analytical runs.

when refrigerated at 5°C were stable for at least 7 weeks. Peak heights of naproxen and MPA in plasma precipitate at 20 and 0.25 μg/ml decreased by 3.4, 1.3 and 4.0% respectively over a 24-h period at 20°C.

3.3. Precision and accuracy

Precision and accuracy of MPA standard curves are listed in Table 2. Standard curves C.V.s ranged from 3.2 to 8.2%. The coefficient of determination (r^2) ranged from 0.9998 to 1.0000 and the mean±S.D. of the slope of all standard curves was 0.1403±0.0092 (6.6%).

3.4. Extraction efficiency

The mean±S.D. absolute recovery of MPA from human plasma was (105.2±9.4)% (n=4 plasma extracts per standard curve concentration). The mean extraction efficiency of naproxen at the concentration employed was 98.9% (n=32 plasma extracts).

3.5. Limit of detection

The limit of detection for MPA in human plasma using the method described was found to be <50 ng/ml.

4. Conclusion

A simple and rapid assay for the determination of MPA in human plasma has been described. The limit of detection, recovery of MPA from plasma and reproducibility were comparable or better than the values obtained using previously developed HPLC methods. The assay is sufficiently robust as to allow the analysis of numerous samples in a single analytical run.

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