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High-performance liquid chromatography determination of mycophenolic acid and its glucuronide metabolite in human plasma

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Abstract

Two HPLC–UV assays are reported here: one is a rapid assay for mycophenolic acid (MPA) and the other is a simultaneous assay for MPA and its metabolite mycophenolic acid glucuronide (MPAG). For both methods, plasma samples (500 μl) with added internal standard were acidified and extracted using C_{18} solid-phase extraction cartridges. Chromatographic separation was achieved on a C_{18} Novapak column using a mobile phase consisting of methanol–0.05% orthophosphoric acid (40:60, v/v) for the rapid MPA assay and 30:70 for the simultaneous MPA and MPAG assay. The assays were linear over the ranges 0.1 to 50.0 mg/l for MPA and 2.8 to 225.8 mg/l for MPAG. Mean absolute recovery for all analytes was >99%. These methods are suitable for therapeutic drug monitoring and pharmacokinetic studies. © 1998 Elsevier Science B.V.

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1. Introduction

Mycophenolate mofetil (MMF, Fig. 1A) is the prodrug of mycophenolic acid (MPA, Fig. 1B), a new immunosuppressive agent which is efficacious in the management of psoriasis [1] and solid organ transplantation [2]. MPA is a reversible, non-competitive inhibitor of inosine monophosphate dehydrogenase (IMPDH), and effectively blocks the de novo pathway of guanosine nucleotide synthesis. A reduction in the guanine nucleotide pool in lymphocytes is the primary consequence of MPA inhibition of IMPDH, which leads to a reduction in DNA synthesis and proliferation of lymphocytes and subsequent immunosuppression [3]. In addition to antilymphocyte activity, MPA prevents arterial smooth muscle cell proliferation, in contrast to conventional immunosuppressants, such as cyclosporin (CsA). This property has potential benefits in opposing obliterative arteriopathy associated with chronic organ rejection [4].

An oral dose of MMF is hydrolysed rapidly during first-pass metabolism to MPA. MPA is metabolised further by conjugation with glucuronic acid forming mycophenolic acid glucuronide (MPAG, Fig. 1C). MPAG may be hydrolysed back to MPA during enterohepatic recirculation [5]. MPA pharmacokinetics are variable in that drug exposure, as measured by area under the concentration–time curve (AUC), is lower in the early post-operative

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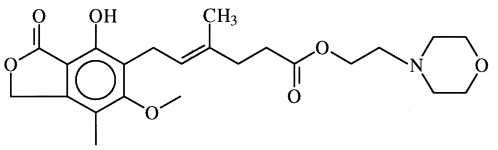
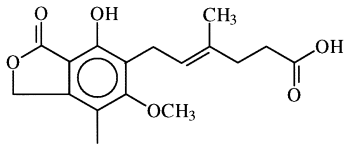
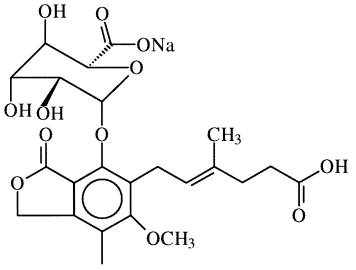


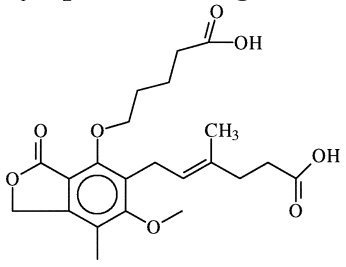
(A) Mycophenolate mofetil (MMF)



(B) Mycophenolic acid (MPA)



(C) Mycophenolic acid glucuronide (MPAG)



(D) Internal standard (IS)

Fig. 1. Structures of (A) mycophenolate mofetil, (B) mycophenolic acid, (C) mycophenolic acid glucuronide and (D) internal standard.

period compared with the late (>20 days) post-operative period. It has been proposed that decreased MPA protein binding results in increased renal clearance early following renal transplantation [6]. Alternatively, or concomitantly, the enterohepatic recirculation process may change over time. Overall, these phenomena may contribute to the inter-patient variability in plasma concentrations observed for MPA.

Clinical trials in renal transplant patients have demonstrated that triple therapy with MMF, CsA and corticosteroids is more efficacious in preventing rejection than the combinations of placebo, CsA and corticosteroids or azathioprine, CsA and corticosteroids. Initial safety data from these clinical trials have demonstrated that adverse gastrointestinal events predominate [7], and that leukopenia and anemia also occur. The long term safety of MMF in transplant patients has not been established.

To assess inter-patient variability and safety, and to target the optimal therapeutic range, assays have been developed to measure MMF [8], MPA and MPAG. Importantly, the published methods for MPA and MPAG have run times unsuitable for high throughput or routine analysis [9], have complicated extraction procedures [10] or require dual analytical columns for MPA and MPAG quantification [11]. In this study, we have developed a high throughput assay for MPA (MPA assay) and a method to determine MPA and MPAG (MPA+MPAG assay) simultaneously using high-performance liquid chromatography (HPLC) with a single analytical column and mobile phase. Importantly, both are technically simple.

2. Experimental

2.1. Materials

MPA, (E)-6-(1,3-dihydro-4-hydroxy-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl)-4-methyl-4-hexenoate; MPAG, (mycophenolic acid glucuronide disodium salt) and internal standard (I.S., Fig. 1D), (E)-6-[1,3-dihydro-4-(4-carboxy-butoxy)-6-methoxy-7-methyl-3-oxo-5-isobenzofuranyl]-4-methyl-4-hexenoic acid, were obtained from Syntex (Palo Alto, CA, USA). All solvents were HPLC grade and all reagents analytical-reagent grade. HPLC quality water was prepared using a Millipore Milli-Q purification system (Millipore, Milford, MA, USA). Solid-phase extraction (SPE) cartridges (Isolute, C_{18}, 200 mg, 3 ml) were obtained from Activon (Sydney, NSW, Australia).

2.2. Chromatographic system

The HPLC system consisted of a Model 6000A pump, a 712 WISP autoinjector, a 484 tunable UV absorbance detector, a Nova-Pak C_{18} column (150 mm×3.9 mm I.D, 4 μm, ambient temperature) and Maxima software (Waters, MA, USA). The mobile phase for the MPA assay consisted of acetonitrile–0.05% orthophosphoric acid (40:60, v/v) and acetonitrile–0.05% orthophosphoric acid (30:70, v/v) for the MPA+MPAG assay. The flow-rate for both assays was 1 ml/min. A 50-μl sample volume was injected onto the column and the eluent was monitored at 254 nm.

2.3. Internal standard

The I.S. was prepared as a stock solution of 100 μg/ml in methanol–water (30:70, v/v). A working I.S. solution was prepared by dilution (1:2) of the stock solution in methanol–water (30:70, v/v).

2.4. Extraction procedures

Plasma samples (500 μl) and I.S. working solution (100 μl) were pretreated with 0.5 *M* hydrochloric acid (2 ml). Samples were vortexed for 1 min and centrifuged for 3 min at 860 *g*. The SPE cartridges were preconditioned with methanol (2 ml) followed by water (2 ml). The sample supernatants were applied to their respective cartridges. The loaded cartridges were washed with 40% 0.05 *M* phosphate buffer (pH 3.0)–60% methanol (1 ml) (MPA assay) or 80% 0.05 *M* phosphate buffer (pH 3.0)–20% methanol (1 ml) (MPA+MPAG assay). The analytes were eluted with 20% 0.05 *M* phosphate buffer (pH 3.0)–80% methanol (2×0.5 ml). The samples were vortexed and dispensed into their respective autosampler vials for analysis.

2.5. Validation of assays

Prior to standard solution preparation and assay validation, plasma from transplant patients receiving other medication (e.g., tacrolimus, CsA) was screened to ensure no interfering peaks were present. The linearity of the MPA and MPA+MPAG assays was assessed on four and three days, respectively. Standard curves for MPA (0.1, 0.5, 1.0, 5.0, 10.0 and 50.0 mg/l) and MPAG (2.8, 5.7, 22.6, 33.9, 79.0 and 225.8 mg/l) were constructed using weighted $1/x^2$ linear regression. Imprecision and accuracy for the MPA assay were determined over four days by analysing three controls (0.8, 25.0 and 40.0 mg/l) in quadruplicate. Imprecision and accuracy for the MPA+MPAG assay were determined over three days by analysing three controls (MPA: 0.8, 20.0 and 40.0 mg/l; MPAG: 6.8, 22.6 and 90.3 mg/l) in quadruplicate. Within-day, between-day and total imprecision were calculated from analyses of variance of the assayed controls using the method of Krouwer and Rabinowitz [12]. Accuracy was determined by expressing the mean assayed result for the control samples as a percentage of the weighed-in concentration. Absolute assay recovery was determined by comparing the peak areas of the extracted samples with the peak areas obtained from direct injections of pure compounds in 80% methanol–20% 0.05 *M* phosphate buffer (pH 3.0).

2.6. MPA and MPAG pharmacokinetic profile

A series of blood samples (pre-dose, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 3, 4, 6, 8, 10, 12 h) were collected over a 12-h period from a 42-year-old, female renal transplant recipient. The patient received 1 g MMF bd. The venous blood samples were collected into Vacutainer tubes containing EDTA. Samples were centrifuged (10 min, 1500 *g*) and the plasma removed and stored at −20°C until analysis. The samples were assayed for MPA (MPA assay) and MPAG (MPA+MPAG assay).

3. Results

3.1. MPA

Fig. 2 shows typical chromatograms for (a) blank plasma, (b) plasma standard (0.5 mg/l) and (c) patient sample (7.1 mg/l). The retention times for I.S. and MPA are 4.5 min and 5.0 min. The assay was linear for MPA over the range 0.1 to 50.0 mg/l [$y=9.87\ (\pm0.16)x+0.76\ (\pm1.0)$, $r\geq0.998$, $n=4$] with a limit of detection of 0.1 mg/l. The imprecision and accuracy of the assay are summarised in

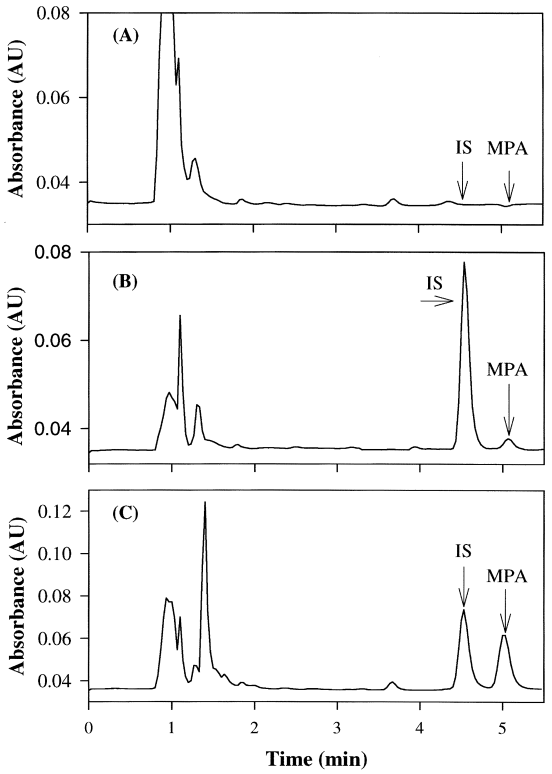


Fig. 2. Typical chromatograms of (A) blank plasma, (B) plasma standard (0.5 mg/l) and (C) a patient sample (7.1 mg/l) for MPA assay. Retention times of I.S. and MPA are 4.5 and 5.0 min, respectively.

Table 1. The total coefficient of variation (C.V.) for MPA was less than 12% for the controls studied. The accuracy of the method for MPA ranged from 97.1% to 98.8%. The absolute recovery of MPA at concentrations of 0.8, 25.0 and 40.0 mg/l is shown in Table 2.

Table 2
Analyte absolute recovery for the MPA and the MPA+MPAG assays

Assay	Weighed-in concentration (mg/l)		Absolute recovery ±S.E.M.[a] (%)	
	MPA	MPAG	MPA	MPAG
MPA	0.8	n/a[b]	109.2±26.1	n/a
(n=16)	25.0	n/a	112.8±15.1	n/a
	40.0	n/a	109.4±10.8	n/a
MPA+MPAG	0.8	6.8	99.9±19.4	107.6±8.2
(n=12)	20.0	22.6	103.4±3.9	107.4±4.2
	40.0	90.3	108.1±5.3	109.3±2.3

[a] S.E.M.=Standard error of the mean.
[b] n/a=Not applicable.

3.2. MPA+MPAG assay

Fig. 3 shows typical chromatograms for (A) blank plasma, (B) plasma standard (79.0 mg/l MPAG; 10.0 mg/l MPA) and (C) patient sample (80.9 mg/l MPAG; 6.2 mg/l MPA). The retention times of MPAG, MPA and I.S. were 2.7 min, 14.8 min and 15.9 min, respectively. The assay was linear for MPA over the range 0.1 to 50.0 mg/l [y=7.80 (±0.19)x+0.04 (±0.02), $r\geq$0.990, n=3]. The assay was linear for MPAG over the range 2.8 to 225.8 mg/l [y=4.92 (±0.17)x−0.65 (±1.5), $r\geq$0.990, n=3]. The limits of detection were 0.1 mg/l (MPA) and 2.8 mg/l (MPAG). The imprecision and accuracy of the assay is summarised in Table 3. The total C.V. for both analytes was less than 15% over the controls studied. The accuracy of the MPA and MPAG assay ranged from 95.7% to 108.7% and 93.8% to 105.1%, respectively. The absolute recoveries of MPA and MPAG, assessed at concentrations of 0.8, 20.0, 40.0

Table 1
Imprecision[a] and accuracy[b] of the MPA assay

Weighed-in concentration (mg/l)	Imprecision (%)			Accuracy (%)
	Intra-day	Inter-day	Total	
0.8	11.2	3.8	11.8	97.1
25.0	1.3	1.6	2.1	98.8
40.0	0.98	0.99	1.4	97.5

[a] Imprecision was calculated by the method of Krouwer and Rabinowitz [12] and expressed in terms of coefficient of variation (n=16).
[b] Accuracy was determined as a percentage of the mean assayed concentration over the weighed-in concentration (n=16).

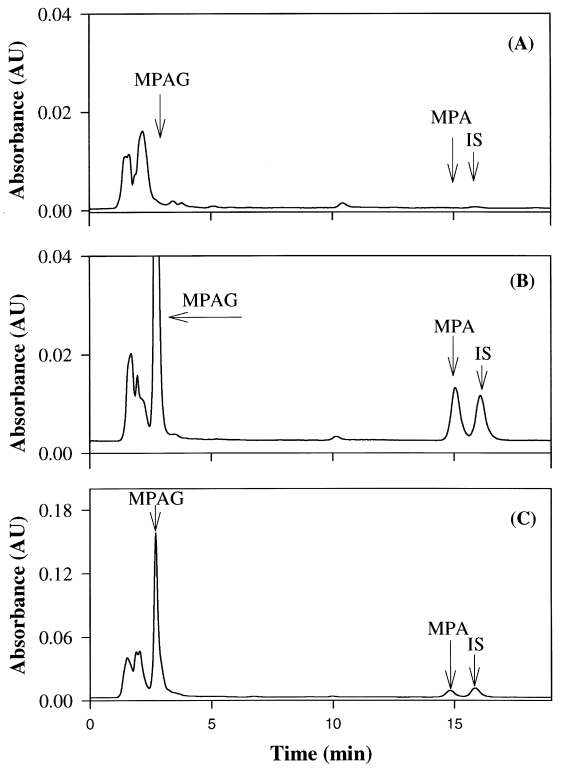


Fig. 3. Typical chromatograms of (A) blank plasma, (B) standard (MPAG: 79.0 mg/l, MPA: 10.0 mg/l) and (C) a patient sample (MPAG: 80.9 mg/l, MPA: 6.2 mg/l) for the MPA+MPAG assay. Retention times of MPAG, MPA and I.S. are 2.7, 14.8 and 15.9 min, respectively.

mg/l and 6.8, 22.6, 90.3 mg/l, respectively, are shown in Table 2.

3.3. MPA and MPAG pharmacokinetic profile

Fig. 4A and Fig. 4B show the MPA and MPAG pharmacokinetic profiles, concentration plotted against time, of a renal transplant patient. Utilising the trapezoidal rule, the area under the curve [$AUC_{(0-12)}$] was calculated to be 28.5 h mg/l (MPA) and 1499 h mg/l (MPAG). MPA clearance was calculated from the ratio of the MMF dose (1 g) to the $AUC_{(0-12)}$ and determined to be 35 l/h. The MPA profile resulting from the MPA+MPAG assay is also shown in Fig. 4A.

4. Discussion

Although a number of published assays exist for the quantification of MPA and MPAG in biological samples most involve lengthy, complex extraction procedures, such as requiring hydrolysis of MPAG to MPA prior to analysis [10], or have chromatographically long run times [9]. A simultaneous MPA and MPAG assay reported previously required two analytical columns and mobile phases to separately quantify MPA and MPAG [11]. The MPA method described here circumvents these problems by using

Table 3
Imprecision[a] and accuracy[b] of the MPA+MPAG assay

Analytes	Weighed-in concentration (mg/l)	Imprecision (%)			Accuracy (%)
		Intra-day	Inter-day	Total	
MPA	0.8	8.1	6.2	10.2	102.5
	20.0	1.2	3.1	3.4	104.4
	40.0	1.9	1.0	2.1	108.7
MPAG	6.8	3.9	14.2	14.7	94.1
	22.6	2.8	4.2	5.1	102.2
	90.3	5.2	1.8	5.5	105.1

[a] Imprecision was calculated by the method of Krouwer and Rabinowitz [12] and expressed in terms of coefficient of variation ($n=12$).
[b] Accuracy was determined as a percentage of the mean assayed concentration over the weighed-in concentration ($n=12$).

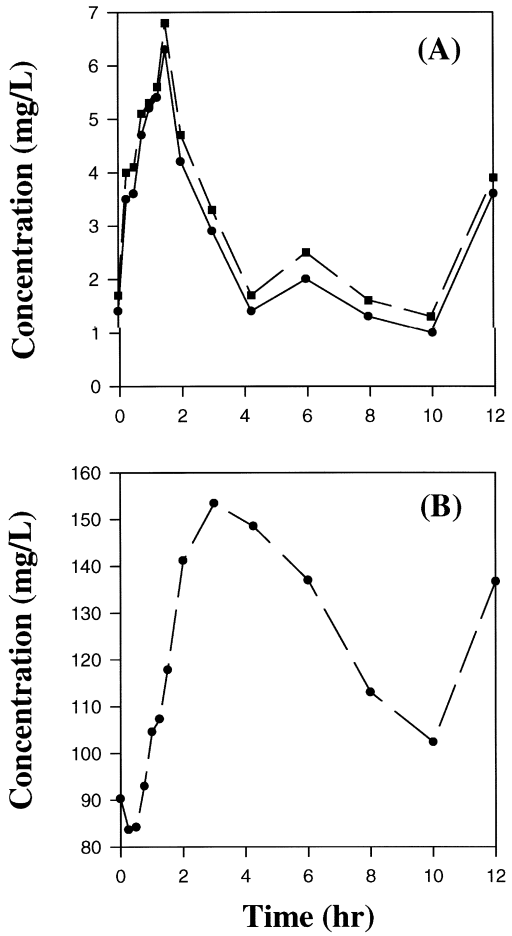


Fig. 4. (A) MPA plasma concentrations of a 42-year-old, female renal transplant recipient after a single 1 g dose of MMF. The solid and dashed lines represent MPA concentration determined by the MPA assay and the MPA+MPAG assay, respectively. (B) MPAG plasma concentrations of the same patient.

a reliable, simple SPE procedure with an analytical column that rapidly resolves both compounds. An analysis time of less than 6 min facilitates a high throughput which is essential for therapeutic drug monitoring. The simultaneous MPA+MPAG assay described here avoids the need to change mobile phase, to use complicated dual column HPLC systems [11] or to enzymatically hydrolyse MPAG to MPA [10]. The analytes are resolved and eluted in under 15 min using a single analytical column and mobile phase whilst retaining the accuracy and precision of the rapid MPA assay. Both methods correspond to a significant saving in time and labour.

MPAG is the major metabolite of MMF and has been reported to have no immunosuppressive activity [13]. MPAG exhibits enterohepatic cycling (EHC) [14], resulting in a secondary peak in MPA concentration. This effect is evident in the MPA pharmacokinetic profile (Fig. 4A) at approximately 6 h post-dose. The EHC of MPAG may explain some of the observed variability in plasma MPA AUC calculations [5]. The MPAG concentrations are at least an order of magnitude greater than the MPA concentrations.

The pharmacokinetic profiles we have shown (Fig. 4A, Fig. 4B) illustrate the usefulness of the MPA and MPA+MPAG assays in obtaining data that can facilitate the study of dose, efficacy and toxicity relationships. The MPA assay presented may be used routinely to individualise dosing and is a reliable method for assessing patient compliance.

References

[1] W.W. Epinette, C.M. Parker, E.L. Jones, M.C. Greist, J. Am. Acad. Determatol. 17 (1987) 962.
[2] H.W. Sollinger, Clin. Transplant. 10 (1996) 85.
[3] J.T. Ransom, Ther. Drug Monit. 17 (1995) 681.
[4] A.C. Allison, E.M. Eugui, Clin. Transplant. 10 (1996) 77–84.
[5] R.E.S. Bullingham, A. Nicholls, M. Hale, Transplant. Proc. 28 (1996) 925.
[6] L.M. Shaw, H.W. Sollinger, P. Halloran, R.E. Morris, R.W. Yatscoff, J. Ransom, I. Tsina, P. Keown, D.W. Holt, R. Lieberman, A. Jaklitsch, J. Potter, Ther. Drug Monit. 17 (1995) 690.
[7] P. Halloran, T. Mathew, S. Tomlanovich, C. Groth, L. Hooftman, C. Barker, Transplantation 63 (1997) 39.
[8] I. Tsina, M. Kaloostian, R. Lee, T. Tarnowski, B. Wong, J. Chromatogr. B 681 (1996) 347.
[9] S. Li, R.W. Yatscoff, Transplant. Proc. 28 (1996) 938.
[10] N. Sugioka, H. Odani, T. Ohta, H. Kishimoto, T. Yasumura, K. Takada, J. Chromatogr. B 654 (1994) 249.
[11] I. Tsina, F. Chu, K. Hama, M. Kaloostian, Y.L. Tam, T. Tarnowski, B. Wong, J. Chromatogr. B 675 (1996) 119.
[12] J.S. Krouwer, R. Rabinowitz, Clin. Chem. 30 (1984) 290.
[13] I. Nowak, J. Wu, K.L. Brayman, L.M. Shaw, Transplant. Proc. 29 (1997) 337.
[14] R. Bullingham, S. Monroe, A. Nicholls, M. Hale, J. Clin. Pharm. 36 (1996) 315.