Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
35 (2004) 87–92
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Development of a high pressure liquid chromatography method for the determination of mycophenolic acid and its glucuronide metabolite in small volumes of plasma from paediatric patients

D.G. Watson [a,*], F.G. Araya [a], P.J. Galloway [b], T.J. Beattie [c]

[a] *Department of Pharmaceutical Sciences, Strathclyde Institute of Biomedical Science (SIBS), University of Strathclyde, 27 Taylor Street, Glasgow G4 0NR, UK*
[b] *Department of Biochemistry, Royal Hospital for Sick Children, Yorkhill, Glasgow G3 8SJ, UK*
[c] *Department of Nephrology, Royal Hospital for Sick Children, Yorkhill, Glasgow G3 8SJ, UK*

Received 28 October 2003; received in revised form 12 November 2003; accepted 14 December 2003

Abstract

In order to facilitate the simultaneous determination of the levels of mycophenolic acid (MPA) and mycophenolic acid glucuronide (MPAG) in plasma samples a step wise gradient high performance liquid chromatography (HPLC) method was developed using UV detection system and naproxen as an internal standard. The analytes were extracted from plasma using Strata-X polymeric solid phase extraction (SPE) cartridges. Separation was achieved within a total chromatographic run time of 18 min at 1.0 ml/min flow rate using a Hv PURITY® C18 column. The method was found to be linear over the concentration range investigated, 1.0–16 μg/ml ($r > 0.99$) for MPA and 10–160 μg/ml ($r > 0.99$) for MPAG. The limit of detection was 0.1 μg/ml for both MPAG and MPA. The intra- and inter-day imprecisions expressed as R.S.D. were 7.8 and 6.6%, respectively, for MPA (1 μg/ml) and 6.2% and 5.6%, respectively, for MPAG (20 μg/ml). The average extraction recovery from plasma was 93.06%, for MPA and 92.41% for MPAG. The method developed was found to be accurate and precise in quantifying the level of MPA and MPAG over a their therapeutic range of concentrations in small volumes of plasma and thus can be effectively used in the routine drug monitoring procedures and pharmacokinetic studies. It was also developed in such a way that it should be easily coupled to an electro-spray ionization mass spectrometer should greater sensitivity be required.

Keywords: Mycophenolic acid; Glucuronide; HPLC; Diode array detection; Solid phase extraction

1. Introduction

Mycophenolic acid (MPA) is a fermentation product of several Penicillium species of fungus. MPA is a potent, selective, uncompetitive, and reversible inhibitor of inosine monophosphate dehydrogenase, and therefore inhibits the de novo pathway of guanosine nucleotide synthesis without incorporation into DNA [1]. Because T- and B-lymphocytes are critically dependent for their proliferation on de novo synthesis of purines, whereas other cell types can utilize salvage pathways, MPA has potent cytostatic effects on

* Corresponding author. Tel.: +44-141-548-2651; fax: +44-141-552-6443.
E-mail address: d.g.watson@strath.ac.uk (D.G. Watson).

0731-7085/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2003.12.011

lymphocytes. MPA also inhibits proliferative responses of T- and B-lymphocytes to both mitogenic and allospecific stimulation.

Mycophenolate mofetil (MMF), the ester pro-drug of MPA, is rapidly and completely converted into the active metabolite form following oral or parenteral administration. The bioavailability of the active substance is around 94% and the maximum plasma concentration of MPA following an oral dose is reached in around 0.8 h. The average half-life for healthy adults is 16 h. The main metabolite of MPA is the pharmacologically inactive 7-*O*-glucuronide (phenolic glucuronide, MPAG), which is formed mainly in the liver and biliary system; up to 87% of MPAG is excreted in the urine. The remainder is secreted into the bile and is subject to enterohepatic circulation [2]. In the intestine deglucuronidation of MPAG to MPA takes place and the de-glucuronidated MPA is then reabsorbed resulting in a second MPA concentration maximum in plasma 6–12 h after the initial intake of MMF. In addition to MPAG, two other metabolites have recently been identified in humans. One is the acyl-glucuronide, which in vitro has pharmacologically activity similar to the parent compound and the other is 7-*O*-glucoside. About 6% of MMF is eliminated unchanged in the stools. At clinically relevant concentrations, 97% of MPA and 82% of MPAG is bound to plasma albumin [3], and over 99.9% of MPA has an extracellular plasma distribution. For this reason, plasma is the preferred medium for measuring the concentration of MPA.

MMF is widely used as an immunosuppressive agent in patients following solid organ transplantation and plasma MPA concentration has been used as an indicator of the risk of development of organ rejection. For this and other reasons monitoring of the plasma level in patients taking MMF is gaining importance. Some of the reasons for monitoring the drug level in plasma include the following.

1. The MPA dose-interval area under the concentration (AUC) time curve is predictive of the risk of development of acute organ rejection if the level is below the optimal therapeutic concentration [4].
2. The highly significant, up to 10-fold inter-individual variation of MPA area under the concentration time curve values, in heart and renal transplant patients receiving a fixed dose of the parent drug.
3. Substantial changes in MPA concentrations can occur in patients receiving a fixed dose of the parent drug as a result of drug–drug interactions due to co-administration with other immunosuppressive agents such as cyclosporine or tacrolimus.
4. The effect of liver and kidney [5,6] diseases on the total and free MPA levels in the body.
5. The need to closely monitor plasma MPA levels when a major change in immunosuppression is planned such as steroid withdrawal.

The focus of the current investigation was on the development of a rapid and sensitive method for the analysis of both MPA and its glucuronide metabolite using a single analytical method.

Although several high performance liquid chromatography (HPLC) methods were developed to analyze MPA and MPAG in human plasma, due to a wide difference in hydrophilicity between the two analytes the earlier developed methods were able to analyse MPA only [7–9]. Alternatively separate HPLC systems were used by changing the analytical columns and mobile phase composition for the analysis of each of the two compounds [10]. To overcome the shortcomings of using two different chromatographic systems, an automated technique that employed robotic operations [10] was suggested, however the system was unnecessarily complex. Other liquid chromatographic methods capable of determining both MPA and MPAG simultaneously have also recently been published [11–16]. Some of these and other published methods use protein precipitation [10–19] less easy to automate than solid phase extraction (SPE) and others use either phosphoric acid, sulphuric acid or phosphate buffer as a component in their mobile phase [10–17] which makes their system unsuitable for coupling to an MS.

Recently, a HPLC–MS–MS method reported a lower limit of quantification for MPA (2.5 ng/ml) [8] than that achievable by HPLC. Although it is possible to analyze components that are not well separated by HPLC using MS–MS, in view of the potential interference of the metabolite in the analysis of the parent drug due to inter-conversion in the mass spectrometer, good HPLC separation is required prior to HPLC–MS–MS analysis [20].

The current paper describes a method for the simultaneously determination of both MPA and MPAG using HPLC with diode array detection.

2. Experimental

2.1. Materials

Formic acid (Analar), ammonia solution, methanol and water (all HPLC grade) were obtained from BDH-Merck, Lutterworth, Leics. Mycophenolic acid and naproxen sodium were from Sigma–Aldrich, Poole, Dorset. Mycophenolic acid glucuronide (MPAG) was supplied by Analytical Services, Caterham, Surrey.

2.2. HPLC equipment

A Thermo Separation Products P4000 pump was used with a vacuum degasser (SCM1000), an AS3000 autosampler and UV6000LP (Photo Diode Array) and X-Calibre software. A Hv PURITY® C18 column (100 mm × 4.6 mm, 5 μm, Thermo Hypersil) fitted with a Security Guard cartridge was used. The mobile phase conditions at a flow rate of 1 ml/min were: methanol: 2 mM ammonium formate pH 3.8 (33:67) to 6 min, methanol: 2 mM ammonium formate pH 3.8 (60:40) at 7 min, methanol: 2 mM ammonium formate pH 3.8 (60:40) to 18 min.

2.3. Collection of samples

Fourteen plasma samples were collected from nine stable post-renal transplant paediatric patients and one patient with nephrotic syndrome. All samples were collected 11–14 h after the latest drug ingestion to ensure a trough level was obtained. Of the nine post transplant patients in addition to MMF, four were on Tacrolimus, four on Cyclosporin and one on Rapamycin. The individual daily dosage of MMF expressed by weight and surface area, are given in Table 1. The plasma was stored at a temperature of −20 °C on receipt and kept at the same temperature until the day of analysis when they were thawed at room temperature.

2.4. Assay validation

2.4.1. Calibration

Aliquots of blank human plasma (100 μl) were spiked with 1, 2, 4, 8, and 16 μg/ml of MPA and 10, 20, 40, 80, and 160 μg/ml for MPAG and the samples were vortex mixed. The samples were then spiked with 20 μg of naproxen sodium internal standard and 200 μl of 0.5 M formic acid were added and the sample was vortex mixed again. Strata-X SPE columns (33 mg, Phenomenex, Macclesfield, UK) were conditioned with 1 ml methanol followed by 1 ml water. The samples were then loaded onto the conditioned SPE. The SPE cartridges were washed with 1 ml 5% methanol and dried for 1 min under vacuum. The analytes retained on the SPE cartridge were then eluted with 2 × 250 μl of 2% formic acid in methanol and the

Table 1
Patient treatments and the associated MPAG and MPA levels in plasma

Patient	Dose per day (mg)	Dose (mg/kg per day)	Dose (mg/M^2 per day)	[MPA] (μg/ml)	[MPAG] (μg/ml)	Other immunosuppressants
1	750	14.88	500	0.865647	18.35196	Tacrolimus
	750	14.82	500	0.441423	12.74746	Tacrolimus
2	1250	26.59	933	4.700517	35.90612	Rapamycin
	1250	25.51	906	10.09595	76.68481	Rapamycin
3	750	16.48	532	4.522402	87.63966	Cyclosporin
	750	16.48	532	6.01813	103.0282	Cyclosporin
4	500	11.82	373	7.40662	132.6706	Cyclosporin
	1000	23.09	741	1.091294	168.5035	Cyclosporin
5	500	26.88	667	0.460648	20.65759	Tacrolimus
6	500	8.77	340	1.138964	57.87702	Cyclosporin
7	750	11.19	478	1.019769	30.44231	Tacrolimus
8	1000	27.02	775	0.397651	128.3459	Cyclosporin
9	1250	16.66	644	7.351241	89.76349	Tacrolimus
10	1500	38.2	1181	8.627179	92.13427	None

solvent was then removed at a temperature of 45 °C under a stream of nitrogen gas. The residue was re-dissolved in 100 μl of mobile phase. The mixture was then transferred into an autosampler vial fitted with a 200 μl glass insert and 20 μl aliquot was injected into the HPLC.

2.5. Intra-day and inter-day precision and accuracy

Five aliquots of blank human plasma (100 μl) were spiked with volumes of standard mixture of MPA and MPAG to yield concentrations of 1 μg/ml MPA and 20 μg/ml MPAG and another five aliquots were spiked at a level of 10 μg/ml MPA and 200 μg/ml MPAG. After addition of 20 μg of internal standard and 200 μl of 0.5 M formic acid and thorough vortex mixing, the mixtures were treated and analysed in the same manner as the calibration series samples. An inter-day precision test was conducted by repeating analysis of these samples on five different days.

2.5.1. Recovery

Plasma was loaded onto Strata SPE columns. The eluates were collected and analysed and the peak areas obtained were compared against those of unextracted standard samples.

2.5.2. Limit of detection

The limit of detection was taken as the concentration of analyte which gave ca. 3× the baseline noise.

2.5.3. Extraction of patient plasma samples

Aliquots of plasma (100 μl) from patients were spiked at a level of 20 μg/ml with naproxen sodium and 200 μl of 0.5 M formic acid was added and after thorough vortex mixing, the mixtures were treated and analysed in the same manner as the calibration series samples. Each sample of patient plasma was analysed in duplicate.

3. Results and discussion

The HPLC eluent was monitored with a diode array detection. For the purposes of quantitative determination of MPAG and MPA we found a selected wavelength of 215 nm gave the best sensitivity. Calibration curves were plotted for the peak areas of MPAG and MPA over the peak area of naproxen against concentration. Calibration was undertaken using simple least squares linear regression and in view of the high R^2 values no weighting function was considered necessary. The calibration lines were linear over the range of the calibration MPAG (slope = 0.435 ± 1.46%, R^2 = 0.992 ± 0.064%, n = 2) and MPA (slope = 0.137 ± 1.46%, R^2 = 0.999 ± 0.0141%).

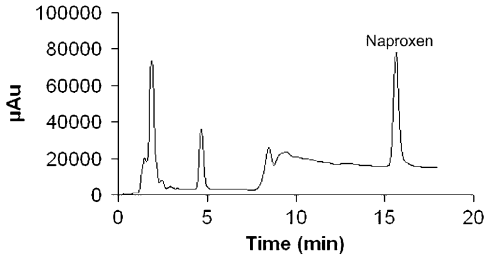

Fig. 1. HPLC chromatogram at 215 nm of blank plasma (100 μl) extracted using SPE.

Fig. 1 shows a chromatogram obtained for blank plasma. There is an interfering peak close to the region in which MPAG is found but Fig. 2 shows an expansion of this region showing that the level of background interference is, in fact, very low. Fig. 3 shows a chromatogram of plasma spiked with 1 μg/ml of MPA and 10 μg/ml of MPAG. The retention times for MPAG, MPA and naproxen were 5.27 ± 0.12, 14.29 ± 0.15 and 15.54 ± 0.16 min, respectively. The values for the intra- and inter-day precisions for MPAG and MPA are shown in Table 2; these precisions are acceptable for a bioanalytical method. The accuracy value for MPAG at a low concentration is high but improves at higher concentration. The accuracy and precision was

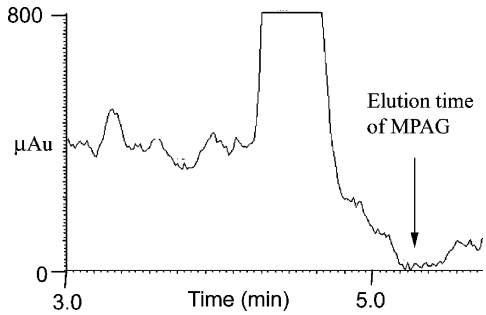

Fig. 2. Expansion of the HPLC trace for an extract from blank plasma in the region where MPAG elutes.

Table 2
Intra- and inter-day precision and accuracy for MPAG and MPA determined in spiked plasma samples

Spiked concentrations	MPAG 20 μg/ml	MPAG 200 μg/ml	MPA 1 μg/ml	MPA 10 μg/ml
Mean amount measured: intra-day ($n = 5$) (μg/ml)	25.8	189.6	1.072	10.29
Mean accuracy: intra-day (%)	129.2	94.8	107.2	102.9
Coefficient of variation: intra-day (%)	±2.4	±6.19	±7.73	±2.53
Mean amount measured: inter-day ($n = 5$) (μg/ml)	24.3	185.0	0.946	10.09
Mean accuracy: inter-day (%)	121.7	92.5	94.6	100.9
Coefficient of variation: inter-day (%)	±5.6	±3.8	±6.6	±2.3

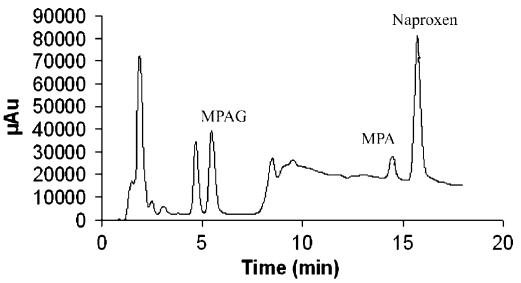

Fig. 3. Blank plasma spiked with 1 μg/ml of MPA and 10 μg/ml of MPAG.

adequate for providing an indication of the relative levels of MPAG and MPA. The lowest concentration that the HPLC method could detect was 0.1 μg/ml for both MPA and MPAG.

Fig. 4 shows a chromatogram of an extract of patient plasma containing a high level of MPAG and a low level of MPA. The MPAG peak is still adequately resolved from the adjacent background peak. The results obtained from the clinical samples are shown in Table 1.

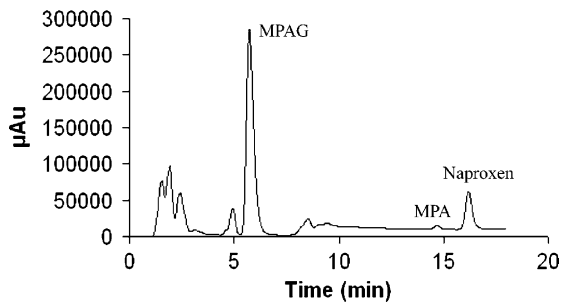

Fig. 4. Extract from patient plasma sample containing high MPAG and low MPA concentrations.

In conclusion, this HPLC method has adequate accuracy and precision for monitoring MPAG and MPA in small volumes of plasma. The method is relatively simple and unlike some previous methods, does not require two different mobile phases for the analysis of MPAG and MPA. The method should be readily coupled to mass spectrometry should higher sensitivity be required. The retention times for MPAG and MPA were about 5.25 and 14.29 min, respectively, and thus each run could be completed within 18 min. A retention time of 5.25 min for MPAG was found to be adequate as the majority of the endogenous peaks of plasma eluted at retention times of less than 3.5 min. Compared to a previously described method [11], using UV monitoring in which the limits of quantification were 0.225 μg/ml for MPA and 9.0 μg/ml for MPAG, the sensitivity of this method showed an improvement. The lower limit of detection of the HPLC method was 0.1 μg/ml for both MPA and MPAG. The 100 μl volume of plasma used for analysis was lower than those required for previous methods which used 200–250 μl [11,21]. No correlation was found between plasma MPA levels and dose and if specific levels are to be achieved, regular monitoring will be required. The low volumes of plasma used in this method are suitable for the analysis of samples from paediatric patients.

References

[1] M.J. Barten, T. Van Gelder, J.F. Gummert, R. Shorthouse, R.E. Morris, Transplant. Immunol. 10 (2000) 1–14.
[2] Y. Matsuzawa, T. Nakase, J. Pharmacobiodyn. 7 (1984) 776–783.
[3] R.E. Bullingham, A.J. Nicholls, B.R. Kamm, Clin. Pharmacokinet. 34 (1998) 429–455.

[4] L.M. Shaw, M. Korecka, D. DeNofrio, K.L. Brayman, Clin. Biochem. 34 (2001) 17–22.
[5] L.M. Shaw, R. Mick, I. Nowak, M. Korecka, K.L. Brayman, J. Clin. Pharmacol. 38 (1998) 268–275.
[6] B. Kaplan, H.U. Meier-Kriesche, G. Friedman, S. Mulgaonkar, S. Gruber, M. Korecka, K.L. Brayman, L.M. Shaw, J. Clin. Pharmacol. 39 (1999) 15–20.
[7] D.A. Saunders, J. Chromatogr. B 704 (1997) 379–382.
[8] C. Willis, P.J. Taylor, P. Salm, S.E. Tett, P.I. Pillans, J. Chromatogr. B 748 (2000) 151–156.
[9] H. Hosotsubo, S. Takahara, Y. Kokado, S. Permpongkosol, J.D. Wang, T. Tanaka, K. Matsumiya, M. Kitamura, A. Okuyama, H. Sugimoto, J. Pharm. Biomed. Anal. 24 (2001) 555–560.
[10] I. Tsina, F. Chu, K. Hama, M. Kaloostian, Y.L. Tam, T. Tarnowski, B. da Wong, J. Chromatogr. B 675 (1996) 119–129.
[11] D. Teshima, K. Otsubo, N. Ktagawa, S. Yoshimur, Y. Itoh, R. Oishi, J. Clin. Pharm. Ther. 28 (2003) 17–22.
[12] G. Seebacher, G. Weigel, E. Wolner, R. Mallinger, M. Grimm, G. Laufer, E.I. Menyawi, A. Griesmacher, M.M. Muller, Clin. Chem. Lab. Med. 37 (1999) 409–415.
[13] K. Na-Bangchang, O. Supasyndh, T. Supaporn, V. Banmairuroi, J. Karbwang, J. Chromatogr. B 738 (2000) 715–720.
[14] J.J. Huang, H. Kiang, T.L. Tarnowski, J. Chromatogr. B 698 (1997) 293–300.
[15] C.E. Jones, P.J. Taylor, A.G. Johnson, J. Chromatogr. B 708 (1998) 229–234.
[16] H. Hosotsubo, S. Takahara, Y. Kokado, S. Permpongkosol, J.D. Wang, T. Tanaka, K. Matsumiya, M. Kitamura, A. Oluyama, H. Sugimoto, J. Chromatogr. B: Biomed. Sci. Appl. 753 (2001) 315–320.
[17] M. Shipkova, P.D. Niedmann, V.W. Armstrong, E. Schutz, E. Wieland, L.M. Shaw, M. Oellerich, J. Clin. Chem. 44 (1998) 1481–1488.
[18] J.F. Gummert, U. Christians, M. Barten, H. Silva, R. Morris, J. Chromatogr. B 721 (1999) 321–326.
[19] W.M. Niessen, J. Chromatogr. A 1000 (2003) 413–436.
[20] M. Vogeser, R. Zachoval, U. Spohrer, K. Hacob, Ther. Drug Monit. 23 (2001) 722–724.
[21] K. Wiwattanawongsa, E.L. Heinzen, D.C. Kemp, R.E. Dupuis, P.C. Smith, J. Chromatogr. B 763 (2001) 35–45.