ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
22 (2000) 197–202

JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS

www.elsevier.com/locate/jpba

Short communication

Method development for the determination of the immunosuppressive drug mycophenolic acid in serum with MECC

S. Noé [a], J. Böhler [b], A.W. Frahm [a,*]

[a] *Chair of Pharmaceutical Chemistry, Pharmaceutical Institute, University of Freiburg, Hermann-Herder-Straße 9, D-79104 Freiburg im Breisgau, Germany*

[b] *Department of Nephrology, University of Freiburg, Hugstetter Straße 55, D-79106 Freiburg im Breisgau, Germany*

Received 20 May 1999; received in revised form 9 August 1999; accepted 25 August 1999

Keywords: Capillary electrophoresis; Mycophenolate; Direct serum injection; Solid phase extraction

1. Introduction

Mycophenolate mofetil (MMF, Fig. 1) (CellCept®), the 2-(4-morpholino)ethylester prodrug of mycophenolic acid (MPA, Fig. 1) is a novel immunosuppressive agent, which is effective in adjunctive therapy with corticosteroids and ciclosporin A for the prevention of graft rejection in patients receiving solid organ transplantation [1–3]. After peroral administration, MMF is rapidly bioactivated to the active species MPA, which is a potent inhibitor of the inosine monophosphate dehydrogenase, the key enzyme of the de novo purine synthesis pathway in lymphocytes.

The determination of serum levels of MPA is especially interesting since MPA undergoes a entero-hepatic circuit as its glucuronide conjugate which is reabsorbed out of the intestine and repasses the organism. Up to date only HPLC-separation procedures and an immunoassay have been described as drug monitoring techniques [4–9]. A method for the determination of MPA using micellar electrokinetic capillary chromatography (MECC) for rapid drug-screening in outpatient's departments with the additional advantage of low-priced and less pollutant chemicals has still to be established.

2. Experimental

2.1. Chemicals

MPA was purchased from Sigma–Aldrich

* Corresponding author. Tel.: +49-761-203-6335; fax: +49-761-203-6351.

E-mail address: awfrahm@ruf.uni-freiburg.de (A.W. Frahm)

0731-7085/00/$ - see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0731-7085(99)00256-3

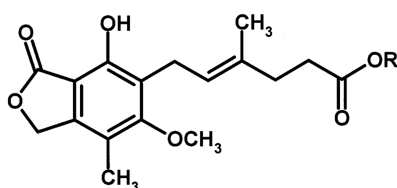

Fig. 1. Structures of MPA and MMF.

(Steinheim, Germany) and sodium dodecyl sulfate (SDS) from BioRad (Richmond, USA). Methanol (Rotisolv®, Roth, Karlsruhe, Germany) and acetonitrile (Janssen, Geel, Belgium) were of HPLC grade. Stock solutions of MPA were prepared in methanol to concentrations of 80, 160, 400, 800, 1600 μg ml^{-1} and stored at ca. 8°C.

2.2. Equipment

MECC was performed on a Prince Crystal CE 310 Capillary Electrophoresis System (ATI Unicam, Kassel, Germany) with autosampler and automatic outlet buffer changer (butler). Separations took place in fused-silica capillaries (Laser2000, Weßling, Germany) with i.d. 50 μm, total length 66 cm and effective length 49.5 cm. Detection was effected with an Unicam 4225 UV-detector (ATI Unicam, Kassel, Germany). Migration time acquisition was performed by an ATI Unicam 4880 chromatography data handling system (version 2.04). Data evaluation and calculation were performed by Microsoft Excel 7.0.

2.3. Separation buffer

All separations were performed using a phosphate–tetraborate separation buffer (pH 8.0, 20 mM) with SDS (50 mM) and acetonitrile (16% v/v). For its preparation 275 mg sodium dihydrogenphosphate hydrate ($NaH_2PO_4 \cdot H_2O$) were solved in 100.0 ml of water (solution 1). 762 mg sodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$) were solved in 100.0 ml of water (solution 2). Solution 2 was added to solution 1 until the pH value reached 8.0 (solution 3). SDS (720 mg) was dissolved in a minimum of solution 3, 8.0 ml acetonitrile were added and the resulting mixture was made up with another portion of solution 3 to 50.0 ml.

Conditioning of new capillaries was effected by rinsing the capillary with 0.1 M NaOH, water, and then with the separation buffer (10 min each). Washes between runs of sample series pretreated with solid-phase-extraction (SPE) were performed by rinsing the capillary with 0.1 M NaOH (10 min), water (10 min) and then with the separation buffer (5 min). In cases of application of the direct serum injection technique (DSI-MECC), washes between runs had to be prolonged to a 30 min treatment with 0.1 M NaOH, while the water and the buffer treatment remained at 10 min each to ensure the complete removal of the serum proteins from the capillary.

2.4. Specimen

Patient and reference blood samples were collected with 10 ml Monovetten® (Sarstedt, Berlin, Germany). The serum was prepared by centrifugation (5000 rpm, 30 min) of the blood samples and stored at ca. −20°C.

2.5. Direct serum injection

Patient serum samples were injected without further pretreatment into the anode capillary inlet as described in Section 2.7.

2.6. Solid phase extraction

Isolute® SPE-columns with C_{18}-endcapped sorbent (ICT, Bad Homburg, Germany) were conditioned with 2 ml of methanol and 2 ml of water. 1000.0 μl serum were acidified with 50.0 μl 1 M HCl and mixed for 10 min. The pretreated serum was applied to the SPE-column and aspirated with a gentle vacuum (ca. −0.05 bar). In follow up the serum vial was washed twice with 1 ml of water and the washing solution was also applied to the SPE-column. The eluates were discarded

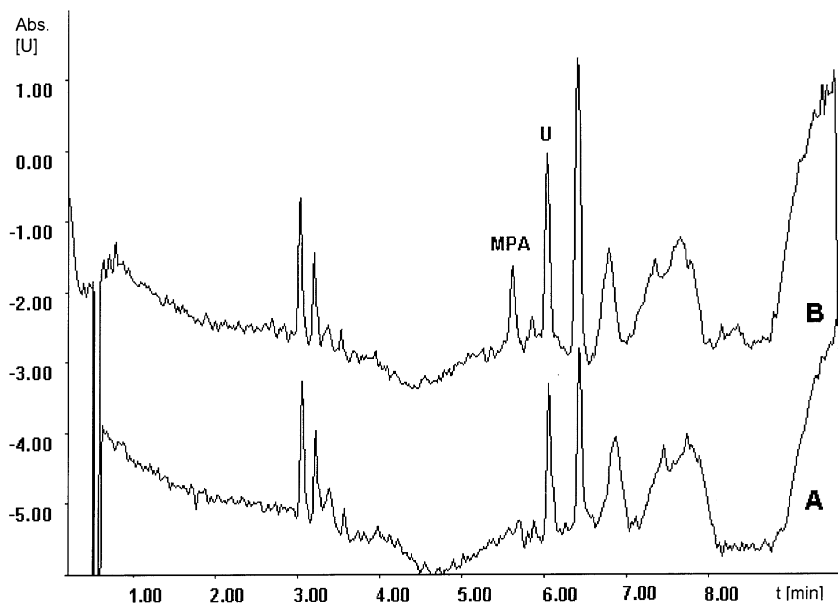

Fig. 2. DSI-MECC electropherograms of serum samples of a transplant patient before (A) and 1 h after peroral administration (B) of 1 g MMF. U, uric acid.

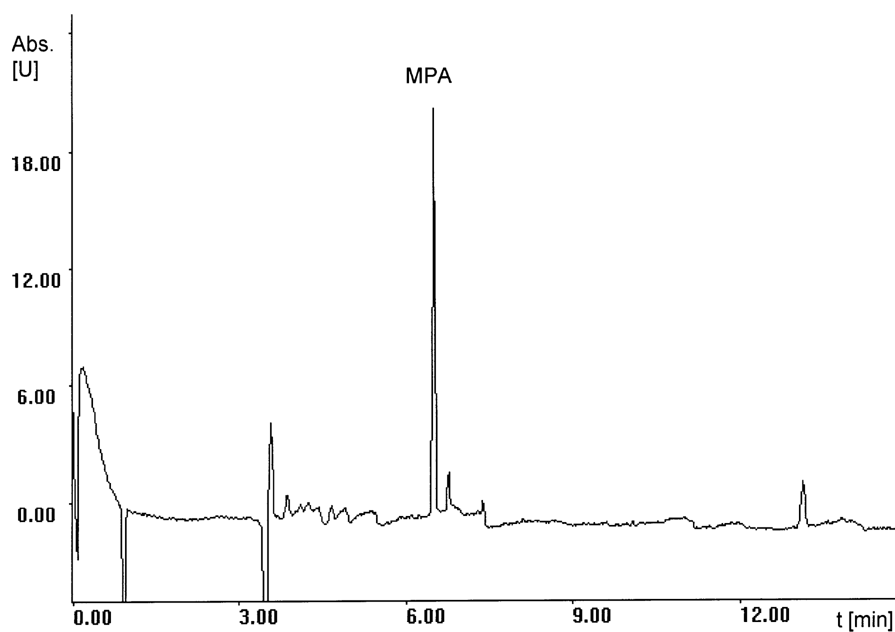

Fig. 3. SPE-MECC electropherogram of a serum containing 20 μg/ml MPA.

and the column was allowed to drip dry. Thereafter the analyte was eluted with 2 ml of acidified methanol (3 ml methanol + 50 μl 1 M HCl). The solvent was removed under a gentle stream of nitrogen and the residue was reconstituted in 50.0 μl of methanol for analysis.

2.7. Analysis

The samples were injected into the anodic capillary inlet under pressure (20 mbar, 0.06 min). The autosampler was held at a temperature of 20 ± 1°C and the capillary oven at 25 ± 0.1°C. Automatic outlet buffer replenishment was performed with the butler. Separations were performed under constant voltage of 30 kV with a current of ca. 65 μA during 10 min. Detection was effected at 254 nm. For quantitative determination of MPA, calibration samples were analysed within the same sample strip together with the serum samples.

3. Results and discussion

3.1. DSI-MECC

MPA can be determined in serum of transplant patients without further sample pretreatment. The drug is released out of its protein binding by the detergent SDS while the endogenous serum proteins are solubilised. Serum samples of a renal transplant patient were drawn before (A) and 1 h after peroral administration (B) of 1 g MMF. Fig. 2 shows the respective electropherograms. In electropherogram B besides MPA endogenous uric acid was detected. The limit of detection for MPA in spiked serum was found at 20 μg ml^{-1} with a rather low but acceptable signal to noise ratio of 2:1 [10,11] which is sufficient for determination of peak levels of MPA in patients under MMF therapy.

3.2. SPE-MECC

With the described SPE-procedure the analyte is concentrated by the factor 20. Under these conditions the limit of quantitation (LOQ) was found at 4 μg ml^{-1} MPA with a signal to noise ratio of 3:1 and a recovery rate of 59%. This LOQ is suitable for monitoring therapeutic trough serum levels of MPA between 4 and 5 μg ml^{-1} [12] during a dose interval after administration of a daily dose of 2–3 g MMF. Fig. 3 shows a typical electropherogram of a SPE-pretreated serum specimen.

3.3. Quantitative determination of MPA using a calibration curve

MPA levels in serum were calculated using peak heights and external standard calibration. Migration times and peak heights of calibration samples are given in Table 1. Reproducibility of the peak heights was much better than that of peak areas and normalized areas. Calibration samples and SPE-treated specimen were analysed within the sample strip in randomized order. The calibration curve was linear in the range of the examined concentrations (80–1600 μg ml^{-1}). These concentrations are equivalent to MPA concentrations gained after a 20-fold concentration of serum with SPE (4–80 μg ml^{-1}) which include the range of expected therapeutic serum levels. The correlation coefficient (r^2) was 0.996.

4. Conclusions

This paper describes the development of separation methods for the determination of MPA in human serum with the highly innovative MECC-technique. No interference was found with the coadministered immunsosuppressive drugs. Corticosteroids do not interfere with DSI-MECC and in low dose therapy with SPE-MECC, whereas in high dose medication steroids are detected with different migration times. Ciclosporin A does not interfere either with DSI-MECC or with SPE-MECC since it is not detected at the wavelength of 254 nm used for monitoring of MPA [11].

The unique technique of direct serum injection (DSI-MECC) for MPA can be used for the rapid compliance control of persons under MMF therapy in outpatients' departments. In contrast, the well-known HPLC technique might have a better limit of detection but suffers from duration of the indispensable sample preparation and high priced solvents. The disadvantage of the commercially available immunoassay are the very high costs. Only with a great sample through-put, immunoassay is a worthwhile rapid method with acceptable costs.

If quantitative determination of MPA is unavoidable, preconcentration of the specimen can

Table 1
Migration times, peak heights and normalized peak areas of calibration samples with RSD's (MPA in methanol)

Concentration [μg/ml]	Migration time (t_m) [min]	RSD of (t_m) [%]	Peak height (h) [units]	RSD of (h) [%]	Normalized peak area (A/t_m) [units]	RSD of (A/t_m) [%]	n
80	7.62	7.4	2.72	7.7	–	–	5
160	7.39	5.1	7.22	4.4	4.80	34.2	5
400	6.32	1.5	21.94	16.4	15.53	13.5	5
1600	6.43	3.9	59.66	21.5	54.52	29.5	5

be performed with the SPE-MECC technique. This sample preparation is easy to perform and does not require more than 1 h. In ongoing studies peak serum levels of MPA are measured in transplant patients with this method.

Acknowledgements

The authors gratefully acknowledge financial support by the Fonds der Chemischen Industrie (Frankfurt am Main, Germany).

References

[1] A.S. Shoker, Drugs Today 33 (1997) 169–174.
[2] A.S. Shoker, Drugs Today 33 (1997) 221–236.
[3] Drugs Fut. 22 (1997) 440–442.
[4] I. Tsina, F. Chu, K. Hama, M. Kaloostian, Y.L. Tam, T. Tarnowski, B. Wong, J. Chromatogr. B 675 (1996) 119–129.
[5] I. Tsina, M. Kaloostian, R. Lee, T. Tarnowski, B. Wong, J. Chromatogr. B 681 (1996) 347–353.
[6] M.M. Müller, M. Vogl, A. Griesmacher, Therapie 52 (1997) 341–344.
[7] E. Schutz, M. Shipkova, V.W. Armstrong, P.D. Niedmann, L. Weber, B. Tonshoff, K. Pethig, T. Wahlers, F. Braun, B. Ringe, M. Oellerich, Transpl. Proc. 30 (1998) 1185–1187.
[8] J.L. Beal, C.E. Jones, P.J. Taylor, S.E. Tett, Ther. Drug. Monit. 20 (1998) 685–690.
[9] J.S. Yeung, W. Wang, L. Chan, Transpl. Proc. 31 (1999) 1214–1215.
[10] S. Noe, J. Böhler, E. Keller, A.W. Frahm, Pharmazie 53 (Suppl.1) (1998) 29.
[11] S. Noe, J. Böhler, E. Keller, A.W. Frahm, Eur. J. Pharm. Sci. 6 (Suppl.1) (1998) S45.
[12] P.J. Smaak Gregoor, T. van Gelder, C.J. Hesse, B.J. van der Mast, N.M. van Besouw, W. Weimar, Nephrol. Dial. Transpl. 3 (1999) 706–708.