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Short communication

Quantification of mycophenolate mofetil in human skin extracts using high-performance liquid chromatography–electrospray mass spectrometry

Manuela Plätzer[a], Konstanze Jahn[a], Johannes Wohlrab[b], Reinhard H.H. Neubert[a,*]

[a]*Martin-Luther-University, Department of Pharmacy, Institute of Pharmaceutics and Biopharmaceutics, Wolfgang-Langenbeck-Strasse 4, 06120 Halle (Saale), Germany*

[b]*Martin-Luther-University, Department of Dermatology, Ernst-Kromayer-Strasse 5–6, 06097 Halle (Saale), Germany*

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Abstract

A sensitive and selective method for the quantification of mycophenolate mofetil and its active metabolite mycophenolic acid in different human skin layers after dermal administration is presented. The skin layers were separated after in vitro penetration experiments and a methanolic extraction was performed. Positive ion electrospray HPLC–MS in selected ion monitoring mode was used to quantify the substances after isocratic separation by a C_{18} analytical column. The minimum detectable concentrations were 850 pg/ml for MMF and 1 ng/ml for MPA. The peak areas depended linearly on the concentration of both drugs over the range of 25–1000 ng/ml ($r^2 \geq 0.996$) with accuracy ≤9.8% and precision ≤13.2%. Total imprecision at quantification limits was 15.2% at 10 ng/ml and 16.3% at 1500 ng/ml for MMF and 15.1% at 21.0 ng/ml and 17.5% at 1300 ng/ml for MPA. This HPLC–MS method will be applicable to the profiling of MMF amounts in skin and its conversion to MPA after application of different formulations.

Keywords: Mycophenolate mofetil

1. Introduction

Mycophenolate mofetil (CellCept®) is an immunosuppressant registered in combination with corticosteroids and cyclosporine A for prevention of acute organ rejection after allogenic heart and kidney transplantations [1].

Mycophenolic acid (MPA), the active metabolite of the prodrug mycophenolate mofetil (MMF) (Fig. 1), provides effective immunosuppression by inhibiting inosine monophosphate dehydrogenase, a key

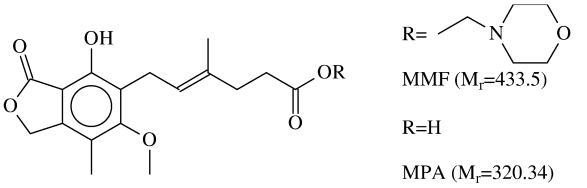

Fig. 1. Structures and molecular masses (M_r) of MMF and MPA.

*Corresponding author. Tel.: +49-345-5525-000; fax: +49-345-5527-292.

E-mail address: neubert@pharmazie.uni-halle.de (R.H.H. Neubert).

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enzyme in the de novo pathway of DNA synthesis that is responsible for guanine production.

Following oral administration, the prodrug MMF is rapidly converted into MPA by plasma and tissue esterases such that no measurable concentrations of MMF have been observed in plasma samples of patients or experimental animals. MPA is primarily metabolised by glucuronidation to the pharmacologically inactive phenolic glucuronide (MPAG), which is the major renal excretion product of the drug [1–3].

The isolation and characterisation of MPA metabolites in plasma of kidney, liver and heart transplant recipients on immunosuppressive therapy with MMF was described using high-performance liquid chromatography (HPLC) and mass spectrometry (MS) [4]. The presence of trace quantities of MPA in MPAG was examined by Korecka et al. using negative ion electrospray HPLC–MS [5].

Recently, several authors have published results concerning the systemic MMF therapy in dermal diseases [6–9]. Adverse effects of MMF such as diarrhoea, nausea, and leucopenia [2] make this administration difficult for dermal therapy. Hence a topical formulation containing MMF should be developed. The aim of this study was the development of a sensitive analytical method to investigate the penetration of MMF into human skin and the possible hydrolysis to the active metabolite (MPA). The method must be able to detect even small amounts of drug in different skin layers after minimal sample preparation.

2. Materials and methods

2.1. Reagents

MMF and MPA were kindly supplied by Hoffmann-La Roche (Basel, Switzerland). Methanol (gradient grade), which was used for sample extraction and as a component of the mobile phase, was purchased from T.J. Baker (Deventer, The Netherlands) and glacial acetic acid from Merck (Darmstadt, Germany). For chromatographic purposes doubly distilled water was applied. The internal standard *n*-hexadecyl-β-D-glucopyranoside (HDG) was supplied by the Department of Pharmaceutical Chemistry, Martin-Luther-University [10,11].

2.2. Sample preparation

Penetration experiments were carried out using Franz-type diffusion cells [12]. The skin samples were obtained from female patients after cosmetic surgery. Prior the preparation of skin the remaining formulation was wiped from the surface (3.14 cm^2) by a cotton wool tip. The different skin layers (stratum corneum, epidermis, dermis) were separated after punch biopsy (total area 0.85 cm^2) by a cryomicrotome (Jung, Heidelberg, Germany). Extraction of the skin layers, the remaining corium and cotton wool tips was performed in Eppendorf tubes or reaction tubes with definite amounts of methanol as shown in Table 1. After vortexing for 1 min the samples were kept in refrigerator overnight. Prior to

Table 1
Preparation of skin samples and cotton wool tip after penetration studies

Sample	Number of sections and thickness	Extraction volume of MeOH (ml)
Stratum corneum	1 section, each 10 μm	1.0
Viable epidermis	4 sections, each 20 μm	1.0
Dermis no. 1	4 sections, each 20 μm	1.0
Dermis no. 2	10 sections, each 40 μm	1.0
Dermis no. 3	10 sections, each 40 μm	0.5
Dermis no. 4	10 sections, each 40 μm	0.5
Remaining corium		1.0
Cotton wool tip		5.0

The cotton wool tip samples were diluted 1:100 with MeOH after extraction.

the analysis the samples were centrifuged for 10 min (5000 *g*) and the supernatant was diluted if necessary with methanol at room temperature. Extracts were measured without further preparation. The aqueous acceptor medium, diluted with methanol, was evaluated, too. A methanolic stock solution (20 μg/ml) of internal standard was prepared. Glucopyranosides as standards have proved reliable in previous work [13] and were chosen to confirm the system stability. A 200 μl portion of the sample or calibration solution and 10 μl of internal standard were placed into vials from which 5 μl was injected. Measurements were performed in triplicate.

2.3. Instrumentation

HPLC was carried out with a Waters 600 E system (Waters, Eschborn, Germany) equipped with a Waters WISP 712 autosampler. This method was performed using a Nucleosil 120-3 C_8 125×2 mm column, 3 μm particle size (Macherey-Nagel, Düren, Germany) with an isocratic mobile phase consisting of methanol–water–glacial acetic acid (80:20:0.02, v/v) at a flow-rate of 0.3 ml/min with a column temperature of 25°C. The HPLC system was coupled to a single quadrupole mass spectrometer Finnigan SSQ 710 C (Finnigan MAT, Bremen, Germany) with an electrospray ionisation (ESI) interface. MS experiments were carried out at a spray voltage of 4.5 kV, and a temperature of 250°C at the heated capillary. Detection was performed in the positive mode. The selected ion monitoring (SIM) mode at *m/z* 434.5 $[M+H]^+$ and 456.5 $[M+Na]^+$ for MMF and 343.4 $[M+Na]^+$ for MPA was used for quantification because of its higher sensitivity. A peakwidth of ±0.5 u and a scan time of 0.25 s were adjusted. The parameters of the ion optic were optimised for MMF.

2.4. Quantification

External standards of MMF or MPA were used for quantification. Stock solutions of both drugs were prepared in methanol and diluted in the range from 0.01 to 1 μg/ml (m/V). These dilutions were measured in triplicate. Quantitation was achieved by plotting the peak area ratio of MMF or MPA to HDG versus the drug concentration followed by linear regression. The calibration curves were accepted for determinations if $r^2>0.996$ and relative standard deviation (RSD)<0.15 for accuracy and precision [14]. To control the system stability and the absence of memory effects a calibration sample (100 ng/ml of each drug) and a blank (methanol) was analysed after every sixth measurement additionally to the internal standard.

3. Results

The composition of the mobile phase was optimised with respect to the sensitivity. An isocratic method was chosen to reduce the influence of changing phases on spray stability and ionisation efficiency. Because of its higher intensity the $[M+H]^+$ ion was used for the quantification of MMF. The ratio between $[M+H]^+$ and $[M+Na]^+$ peak areas of MMF was used to control system stability. Under the given ESI conditions MPA showed only a weak signal for $[M+H]^+$, therefore only the $[M+Na]^+$ signal was monitored for the quantification. The SIM mode provides the highest sensitivity for quantification. Furthermore, a complete separation of the skin components from the drugs was not necessary because co-eluting isobaric matrix interferences were absent. This was tested in separate runs using skin samples treated with formulations containing no drugs (matrix blank). Fig. 2 shows such typical chromatograms of drug determination from skin extracts. Untreated skin samples spiked with MMF or MPA showed recovery rates of more than 92%. The loss is more due to the absorption to the skin than to ion suppression caused by skin components. The recovery rates of MMF from the cotton wool tips were tested by the application of definite amounts of formulation (2–10 mg) to the tips. The recovery rates were always between 80.5 and 84.7%. The analytical parameters observed are listed in Table 2. Both the intra- and inter-assay accuracy and precision were within 13.5% of the theoretical value at each concentration over the entire concentration range, except for the lowest concentrations (10 ng/ml) which had a precision (RSD) of 15.2% (MMF) and 18.7% (MPA). The higher limit of detection was

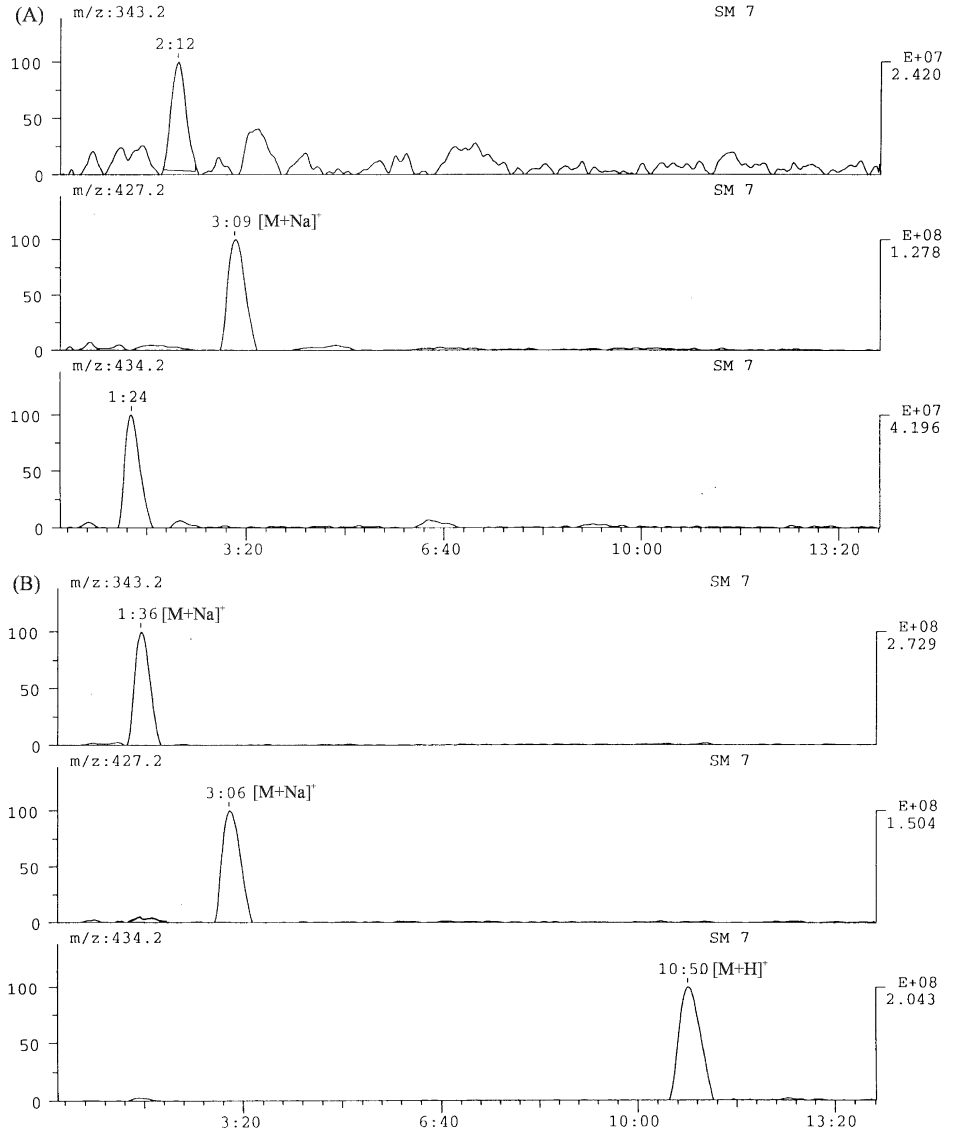

Fig. 2. Selected ion monitoring chromatograms of dermis samples: (A) blank, (B) spiked with MPA and MMF, 150 ng/ml each. The mass ranges stand for MPA (top; 343.2) the internal standard (HDG; 427.2) and MMF (bottom; 434.2). *x*-Axis: retention time (min). *y*-Axis: normalised scan intensity in percent. Peak labelling: retention time. Smooth factor 7.

Table 2
Analytical parameters of both drugs

	MMF $[M+H]^+$	MPA $[M+Na]^+$
Limit of detection[a] (ng/ml)	0.85	1.0
Lower limit of quantification[b] (ng/ml)	10.0	21.0
Higher limit of quantification[b] (μg/ml)	1.5	1.3

[a] Limit of detection: signal-to-noise ratio of 3/1.
[b] Lower and higher limits of quantification: 0.15<variation coefficient from nominal value<0.20 ($n=6$).

determined by the measurement of standards above 1000 ng/ml (steps of 100 ng/ml) of each drug. The limit was reached if the RSD was above 15%. The inter-day RSD calculated from the peak areas of the internal standard was 6.86% (n=90). An improvement of analytical parameters could be reached by the use of deuterated MMF as well as MPA as internal standards, which will show the same ionisation ability. But these substances were not available.

Survey scans of dermis samples showed the $[M+H]^+$ ion of 2-morpholino-ethanol at 132.1 as well as the $[M-H_2O+H]^+$ ion at 114.1. This confirms that MMF hydrolyses to MPA and 2-morpholino-ethanol which was not determined by the following quantifications. Increasing amounts of MPA could be detected in the epidermal and dermal skin layers where the enzymes are situated.

4. Conclusions

In this study a simple but reliable HPLC–MS method has been developed to determine the amount of MMF in different skin layers. Additionally the active metabolite MPA was separated by this method and a simultaneous quantification was possible. The developed method provides a wide range of quantification and a good limit of detection. Liquid scintillation counting would be another possibility to detect this drug in skin samples, but radiolabelled MMF was not available and metabolites cannot be measured by this method. A paper dealing with the penetration results obtained from different pharmaceutical formulations is in preparation.

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References

[1] Product Information – Cellcept, Hoffmann-La Roche, Grenzach-Wyhlen (1996).
[2] B. Fulton, A. Markham, Drugs 51 (1996) 278.
[3] J.J. Lipsky, Lancet 348 (1996) 1357.
[4] M. Shipkova, V.W. Armstrong, E. Wieland, P.D. Niedmann, E. Schütz, G. Brenner-Weiβ, M. Voihsel, F. Braun, M. Oellerich, Br. J. Pharm. 126 (1999) 1075.
[5] M. Korecka, D. Nikolic, R.B. van Breemen, L.M. Shaw, Clin. Chem. 45 (1999) 1047.
[6] C.C. Geilen, H. Orfanos-Boeckel, G. Offermann, C.E. Orfanos, Hautarzt 51 (2000) 63.
[7] M.G. Haufs, S. Beissert, S. Grabbe, B. Schütte, T.A. Luger, Br. J. Dermatol. 138 (1998) 179.
[8] J.E. Silverman Kitchin, M. Keltz Pomeranz, G. Pak, K. Washenik, J.L. Shupack, J. Am. Acad. Dermatol. 37 (1997) 445.
[9] M.G. Hauls, S. Beissert, S. Grabbe, B. Schütte, T.A. Luger, Br. J. Dermatol. 138 (1998) 179.
[10] F. Wilhelm, S.K. Chatterjee, B. Rattay, P. Nuhn, R. Benecke, J. Ortwein, Liebigs Ann. 9 (1995) 1673.
[11] R. Wolf, K. Raith, R. Neubert, J. Chromatogr. A 766 (1997) 71.
[12] T.J. Franz, J. Invest. Dermatol. 64 (1975) 190.
[13] R. Wolf, Ch. Huschka, K. Raith, W. Wohlrab, R. Neubert, Anal. Commun. 34 (1997) 335.
[14] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, Th. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, J. Pharm. Sci. 81 (1992) 309.