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Investigation of the penetration behaviour of mycophenolate mofetil from a semisolid formulation into human skin ex-vivo

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Abstract

Mycophenolate mofetil, the morpholinoethylester of mycophenolic acid, is an immunosuppressant used in combination with ciclosporin (cyclosporin) and corticosteroids to prevent organ rejection after heart and kidney transplantations. The drug seems also to be effective in dermal diseases after systemic administration. However, up to date mycophenolate mofetil can be only systemically administered and this is associated with several side effects such as nausea, leucopenia, sepsis, and diarrhoea. The aim of this study was to develop a topical formulation containing mycophenolate mofetil and to investigate in-vitro release and penetration into human skin ex-vivo. HPLC was applied to quantify mycophenolate mofetil after release studies from semisolid formulations using a dodecanol–collodion membrane as a lipophilic acceptor. Penetration studies with an amphiphilic cream using excised human breast skin were carried out in Franz-type diffusion cells. Mycophenolate mofetil and its active metabolite mycophenolic acid were detected by HPLC-MS after microsectioning in different skin layers. In this study the penetration of mycophenolate mofetil from an amphiphilic cream into excised human skin was shown. Additionally, the enzymatic hydrolysis of penetrated mycophenolate mofetil into mycophenolic acid was proven even under ex-vivo conditions. In-vivo a higher extent of metabolism of mycophenolate mofetil to mycophenolic acid would be expected because of the complete enzyme activity. This topical formulation might be a promising alternative to the usual systemic administration of mycophenolate mofetil in the treatment of skin diseases such as psoriasis.

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Introduction

Mycophenolate mofetil (Figure 1) is an immunosuppressive agent registered in combination with ciclosporin (cyclosporin) and corticosteroids for the prevention of organ rejection after allogenic heart and kidney transplantations. It is the morpholinoethylester prodrug of mycophenolic acid (Figure 1), a fermentation product of several *Penicillium* species. Following oral administration mycophenolate mofetil is absorbed well and is rapidly hydrolysed into its active metabolite mycophenolic acid, which is primarily renally eliminated as the inactive mycophenolic acid glucuronide (CellCept 1996; Fulton & Markham 1996; Lipsky 1996).

Mycophenolic acid is a non-competitive, reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH), a key enzyme of the de-novo-synthesis of guanosine nucleotides required for DNA and RNA generation. In contrast to other

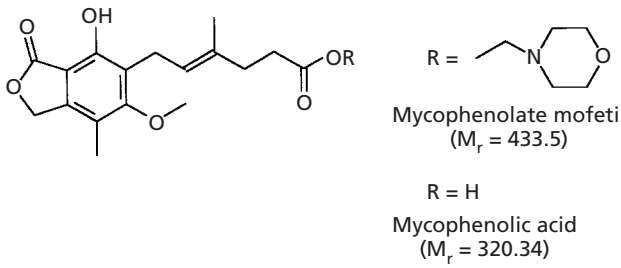

Figure 1 Structure and molecular mass (M_r) of mycophenolate mofetil and mycophenolic acid.

cell types, which can use the salvage pathway for generation of guanosine nucleotides, T- and B-lymphocytes are dependent on de-novo-synthesis. Consequently, an inhibition of IMPDH stops the proliferation of lymphocytes. Mycophenolate mofetil is sparingly soluble in water (0.043 mg mL^{-1} at pH = 7.4). Increasing solubility is observed with decreasing pH value (4.27 mg mL^{-1} at pH = 3.6) (CellCept 1996). Recently, reports have been published concerning the use of mycophenolate mofetil for the treatment of several autoimmune and inflammatory skin disorders such as psoriasis, pemphigus vulgaris and pyodermia gangrenosum (Haufs et al 1998; Grundmann-Kollmann et al 1999; Nousari et al 1999; Geilen et al 2000). The systemic administration in dermal therapy is limited due to the side effects nausea, leucopenia, sepsis, and diarrhoea. Provided that the substance is capable of penetrating into human skin a formulation for the local treatment of skin diseases might be advantageous.

Therefore, the purpose of this study was the development of a suitable topical formulation containing mycophenolate mofetil and the investigation of in-vitro release and penetration into human skin under ex-vivo conditions.

Materials and Methods

Reagents

Mycophenolate mofetil and mycophenolic acid were kindly provided by Hoffmann-La Roche (Basle, Switzerland). Dodecanol (Merck-Schuchardt, Hohenbrunn, Germany) and collodion 4% w/w (Caesar & Loretz GmbH, Hilden, Germany) were necessary to produce the membranes for the release studies. Methanol and acetonitrile (both gradient grade) were purchased from Baker (Deventer, The Netherlands), ethanol from Laborchemie Apolda (Apolda, Germany) and dichlormethane from Grüssing GmbH (Filsum, Germany). Glacial acetic acid and conc. phosphoric acid were obtained from Merck (Darmstadt, Germany). Water was bidistilled quality. Glycerol (Merck, Darmstadt, Germany), isopropylpalmitate, oleic acid and propylene glycol (all Caesar & Loretz GmbH, Hilden, Germany) were used for the solubility determinations. The buffer solutions were prepared using analytical grade substances (Merck, Darmstadt, Germany) according to Rauscher et al (1972). Mc Ilvain buffer consisted of citric acid and disodium phosphate. Sorensen's citrate buffer was prepared from citric acid and sodium hydroxide. The creams were purchased from Pharmazeutisches Kontrollabor (Halle, Germany).

Solubilization studies

For determination of the solubility of mycophenolate mofetil the drug was added in excess to 1.0 mL of each medium to achieve a sediment. The samples were shaken for 1 h and kept in a water bath at 32 ± 1°C over 24 h. After centrifugation (5000 *g*, 10 min) the supernatant was diluted with methanol if necessary and analysed for drug content by high-performance liquid chromatography (HPLC). The experiments were performed in triplicate.

Release studies

Mycophenolate mofetil (2%) was incorporated in three different semisolid formulations: a lipophilic (Unguentum alcoholum lanae aquosum) and a hydrophilic (Unguentum emulsificans aquosum) vehicle according to the *German Pharmacopoeia* (2000) and an amphiphilic vehicle (Cremor basalis) according to the *Deutscher Arzneimittel-Codex* (2000).

In-vitro release studies were performed using a multilayer membrane system (Neubert et al 1991), where four dodecanol–collodion membranes (4% dodecanol w/w) acted as acceptors. A defined amount of each formulation (10–12 mg) was applied on the membrane surface (4.0 cm^2). The models were incubated at 32 ± 1°C over different time intervals (30, 100, 300 and 1000 min). At the end of the experimental periods the remaining formulation was wiped by a swab, the membranes were separated and extracted in reaction tubes.

For the amphiphilic and the hydrophilic vehicles, the membranes were shaken with 5.0 mL ethanol for 30 min. After removal of the membranes these samples were directly analysed for drug content by HPLC.

For the extraction of mycophenolate mofetil after application of the lipophilic cream 5.0 mL dichlormethane was added to the membranes. After shaking (60 min) and removing the membranes the organic solvent was evaporated. The residue was reconstituted with 5.0 mL ethanol, heated in a water bath for a short while to dissolve the cream compounds and shaken for 10 min. These samples were then injected onto the column.

To control the drug content 10–15 mg of the formulations (weighed exactly) were extracted with 5.0 mL ethanol or dichlormethane under the same conditions as described above. The release studies and the drug content analysis were repeated five times.

HPLC was carried out on a Merck-Hitachi-System equipped with a reversed phase column (LiChrospher 60 RP select B, 5-μm particle size, 125×4 mm; both Merck, Darmstadt, Germany) at a flow rate of 1 mL min^{-1}, $\lambda = 214$ nm. A 20-μL sample was injected onto the column. The mobile phase consisted of acetonitrile/water/conc. phosphoric acid (75:25:0.1 v/v/v). The column was equilibrated at 60°C.

Mycophenolate mofetil as external standard was used to generate the calibration curves. A stock solution in ethanol or methanol (solubilization studies) was diluted in the range from 10 to 200 μg mL^{-1}. Quantification was achieved using area vs concentration curves followed by linear regression ($r^2 \geqslant 0.996$).

Penetration studies

Penetration experiments were carried out using Franz-type diffusion cells (Crown Glass Company, Somerville, NJ) (Franz 1975). The studies were performed in triplicate using skin samples from three different patients. Human breast skin from female patients (age 40, 40 and 53) was obtained after cosmetic surgery. After cleaning with 0.9 % sodium chloride solution and removal of the subcutaneous fat, the skin samples were stored at −20°C until use. Before the experiments the skin samples were thawed and placed onto filter gauze in the diffusion cells. The dermal side of the skin was in contact with the acceptor solution (bidistilled water, 20.0 mL) which was stirred continuously. A defined amount of the formulation (15–20 mg) was applied onto the skin surface (3.14 cm^2). The studies were performed under finite dose conditions (Franz 1978). After incubation (30, 300 and 1000 min) at 32 ± 1°C the remaining formulation was wiped by a cotton wool tip. Three punch biopsies (each 0.2827 cm^2) were excised from each skin sample and cut in horizontal sections using a cryomicrotome (Jung, Heidelberg, Germany) as described in Table 1. Several sections were pooled to one sample to guarantee the detection of small drug amounts in the skin. The collected cuts were placed in Eppendorf tubes and extracted with definite amounts of methanol. The tubes were vortexed for 1 min and stored in a refrigerator overnight. The supernatant was diluted with methanol after centrifugation (5000 *g*, 10 min) if necessary. The cotton wool tips were extracted in reaction tubes with methanol. The amount in the different skin layers, in the cotton wool tips and in the acceptor solution was analysed by HPLC-mass spectrometry (HPLC-MS) (Plätzer et al 2001). HPLC was performed on a Waters 600 E system (Waters, Eschborn, Germany) coupled to a single quadrupole mass spectrometer Finnigan SSQ 710 C (Finnigan MAT, Bremen, Germany) with an electrospray ionization interface.

Table 1 Number of sections and thickness of skin samples after penetration experiments.

Skin sample	Number of sections and thickness
Stratum corneum	1 section (10 μm)
Viable epidermis	4 sections (20 μm each)
Dermis 1 (DR 1)	4 sections (20 μm each)
Dermis 2 (DR 2)	10 sections (40 μm each)
Dermis 3 (DR 3)	10 sections (40 μm each)
Dermis 4 (DR 4)	10 sections (40 μm each)
Remaining corium[1]	

[1]Not included in the calculations.

Briefly, electrospray HPLC-MS was used to quantify mycophenolate mofetil and mycophenolic acid following separation on a C8 column with an isocratic mobile phase consisting of methanol/water/glacial acetic acid 80:20:0.02 (v/v/v) at a flow rate of 0.3 mL min^{-1}. The detection was performed in the positive ion mode at m/z 434.5 $[M+H]^+$ for mycophenolate mofetil and 343.4 $[M+Na]^+$ for mycophenolic acid. The detection limit (signal-to-noise ratio of 3:1) was 0.85 ng mL^{-1} for mycophenolate mofetil and 1.0 ng mL^{-1} for mycophenolic acid.

Results

Solubility studies

Solubility of mycophenolate mofetil in different media was determined to obtain more information about the physicochemical properties of the drug. The results are

Table 2 Solubility (c_S) of mycophenolate mofetil in different media (data are means ± s.d.; n = 3) (Fischer 2000).

Medium	c_S (mg mL^{-1})
Ethanol	16.14 ± 2.75
Glycerol	1.24 ± 0.51
Isopropylpalmitate	3.69 ± 0.56
Oleic acid	40.62 ± 1.27
Propylene glycol	5.94 ± 1.43
Mc Ilvain buffer pH = 5	3.33 ± 0.61
Mc Ilvain buffer pH = 6	0.67 ± 0.17
Sorensen's citrate buffer pH = 5	5.08 ± 1.37
Sorensen's citrate buffer pH = 6	1.94 ± 0.64
Bidistilled water	0.21 ± 0.07

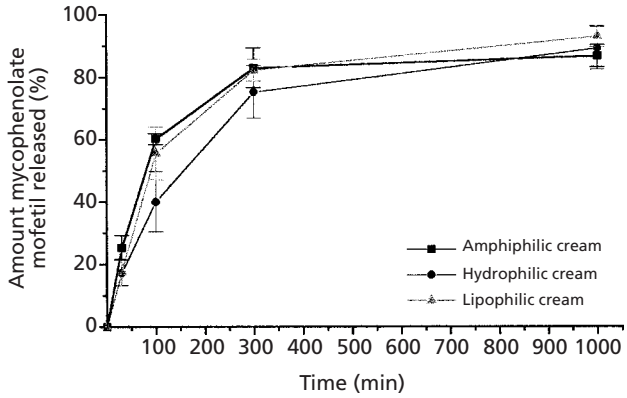

Figure 2 Time dependent in-vitro release of mycophenolate mofetil from three different semisolid formulations using lipophilic membranes as acceptor. Each value represents the total drug content of the four membranes (data are means ± s.d., n = 5).

summarized in Table 2. As published in the literature (CellCept 1996), increasing solubility with decreasing pH was confirmed to be caused by a protonation of the morpholine. The best solubility was observed in oleic acid, whereas the amount of mycophenolate mofetil solubilized in propylene glycol was acceptable as well.

Release studies

The results of the in-vitro release are demonstrated in Figure 2. However, the release of mycophenolate mofetil was sufficient from all vehicles tested. Approximately 25% of the applied dose was released from the amphiphilic vehicle after 30 min. The amount of mycophenolate mofetil in the membranes increased rapidly up to 60% within 100 min. Comparing the drug release of all formulations no further increase could be observed between 300 and 1000 min (Fischer 2000). These results indicated that the drug release was not the rate-limiting

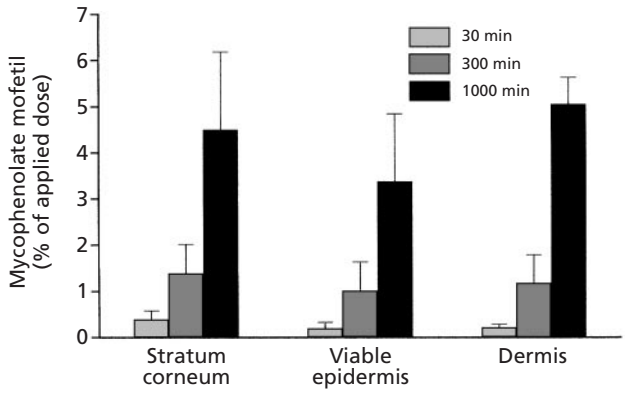

Figure 3 Penetration of mycophenolate mofetil from an amphiphilic vehicle into human skin under ex-vivo conditions (% of applied dose, data are means ± s.d., n = 3), DR 1–4 are summarized as dermis.

step for the penetration of mycophenolate mofetil into human skin.

Only one formulation could be considered for the penetration experiments owing to the limited availability of excised human skin. The amphiphilic cream was selected for further investigation because this formulation showed a marginally higher drug release after 30 and 100 min and may exert penetration enhancing properties caused by the propylene glycol content. Due to the good solubility in propylene glycol (see Table 2) a promoted drug penetration of mycophenolate mofetil into or permeation through human skin as a 'solvent drag' is supposed (Barry 1987, 1991; Bendas et al 1995).

The drug stability in the amphiphilic cream was tested over at least 12 months. No changes in drug content in this formulation could be observed.

Penetration studies

The amount of mycophenolate mofetil (expressed as % of applied dose) that penetrated the stratum corneum, viable epidermis and dermis is summarized in Figure 3. The penetrated mycophenolate mofetil was distributed in approximately equal quantities in the skin layers independent of the incubation time.

The concentration profile of mycophenolate mofetil in the different skin layers is presented in Figure 4. After 30 min, mycophenolate mofetil was detectable only in the stratum corneum and no drug transport into deeper skin layers occurred. As expected, the highest drug concentration was observed in the stratum corneum. Due to its lipophilic properties the substance had a high affinity to this skin layer and a considerable depot was formed from which the drug diffused slowly into deeper parts of the skin. Comparing all times the drug dis-

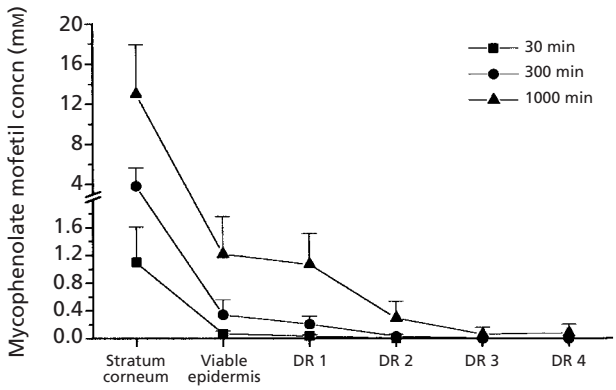

Figure 4 Concentration of mycophenolate mofetil (mM) in different skin layers after application of an amphiphilic cream (data are means ± s.d., n = 3). DR 1–4 = different dermis sections (see Table 1).

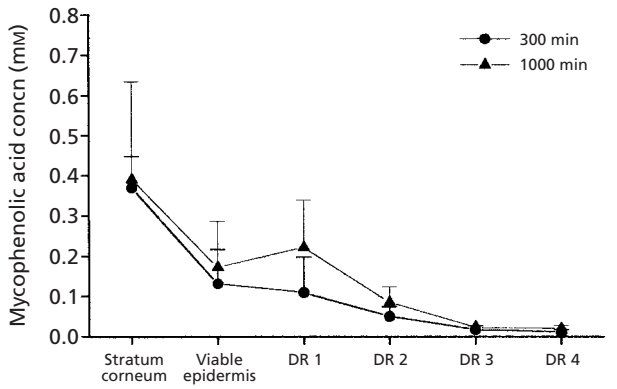

Figure 6 Concentration of mycophenolic acid (mM) metabolized from mycophenolate mofetil in different skin layers after 300 and 1000 min (data are means ± s.d., n = 3). DR 1–4 = different dermis sections (see Table 1).

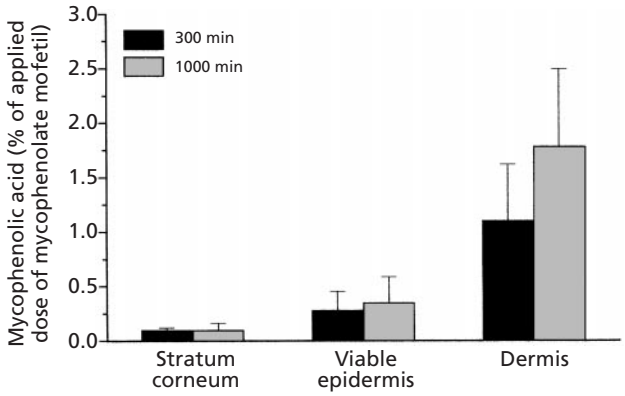

Figure 5 Metabolized amount of mycophenolic acid after 300 and 1000 min (% of applied dose of mycophenolate mofetil; data are means ± s.d., n = 3).

tribution between stratum corneum and the viable epidermis remained the same.

In contrast to 30 and 300 min, a small amount of mycophenolate mofetil (0.1% of applied dose) was found in the acceptor solution after 1000 min. This confirmed the observation that mycophenolate mofetil permeated very slowly from the viable epidermis into the deeper dermis.

An interesting fact of these examinations was the detection of the active metabolite mycophenolic acid in human skin. As mentioned above, the mycophenolic acid content in the different skin layers was also determined by HPLC-MS. As a prodrug, mycophenolate mofetil was hydrolysed rapidly in-vivo to mycophenolic acid by plasma or tissue esterases. The mycophenolic acid amounts arising from the metabolism of penetrated mycophenolate mofetil are presented in Figure 5. After 30 min mycophenolic acid was found in none of the skin layers. The detected amounts of mycophenolic acid were very small because of limited enzyme capacity in excised human skin. As shown in Figure 6, the concentration profiles between 300 and 1000 min were similar, which could have been caused by reduced esterase activity. Mycophenolic acid was mainly detected in the viable epidermis and the upper dermis (DR 1) where the hydrolysing enzymes were located. This observation was in accordance with other published research that has reported that the highest expression of xenobiotic metabolizing enzymes can be found in the viable epidermis and the upper dermis (Steinsträsser & Merckle 1995; Hotchkiss 1998).

Usually, a metabolism in the stratum corneum is impossible. The concentration of mycophenolic acid in the stratum corneum could be explained by a redistribution of metabolized mycophenolic acid from deeper skin layers following a concentration gradient. The results confirmed the assumption that a certain extent of drug metabolism is even possible in human skin under ex-vivo conditions.

No mycophenolic acid was detected in the acceptor solution. To exclude a chemical degradation of mycophenolate mofetil to mycophenolic acid out of the skin the applied formulation and the cotton wool tips were examined for mycophenolic acid content. No mycophenolic acid was found in either sample.

Considering the fact of limited metabolism in excised skin a higher conversion rate from mycophenolate mofetil to mycophenolic acid will be expected in-vivo. It is supposed that a metabolism of mycophenolate mofetil into its active form in human skin is the prerequisite for

the effectiveness of topically applied mycophenolate mofetil in dermal diseases.

Discussion

In this study in-vitro release and penetration of mycophenolate mofetil from an amphiphilic cream into human skin ex-vivo were examined. It was demonstrated that mycophenolate mofetil was released from this vehicle and was capable of penetrating into human skin. Furthermore, the biotransformation of the prodrug to its active form in the skin could be detected. The metabolic capacity of the skin was used for the conversion of a prodrug following percutaneous absorption to a pharmacologically active substance.

Due to the up-regulation of enzyme activity in skin of psoriasis patients it is assumed that the conversion of mycophenolate mofetil to its active metabolite occurs to a higher extent than in healthy skin and in human skin ex-vivo. Penetration experiments showed that only small amounts of mycophenolate mofetil and mycophenolic acid, respectively, were found in deeper skin layers (DR 2–4). Therefore, it would seem that only small quantities of both substances reached the systemic circulation. Consequently, side effects via this administration route could be avoided.

Recently, Wohlrab et al (2001) performed a case study concerning the topical application of mycophenolate mofetil in three patients with plaque-type psoriasis. In those examinations topically applied mycophenolate mofetil showed the same effect as 0.1 % betamethasone-17-valerate cream in reduction of erythema, infiltration and desquamation. However, those observations have to be confirmed in clinical trials. Furthermore, the results of this study suggest that the use of mycophenolic acid in dermal therapy should be reconsidered.

Biotransformation by the skin can lead to an inactivation of the parent drug or to the production of pharmacologically active metabolites. Generally, the extent of metabolism in the skin is smaller than in the liver. According to Täuber (1982) the metabolic clearance of total skin would not exceed 0.8 % of that of the liver.

Information about the skin as a drug metabolizing organ is summarized in several articles (Guy et al 1987; Bronaugh et al 1989; Boehnlein et al 1994; Hotchkiss 1998). Studies have been performed to estimate the importance of cutaneous metabolism of xenobiotics e.g. after topical application of corticosteroids (Gysler et al 1999), propranolol (Ademola et al 1993) and glyceryl trinitrate (Higo et al 1992).

Ademola et al (1993) investigated the metabolism of topically applied propranolol during percutaneous absorption in human skin from different sources. In those studies propranolol metabolism in excised and in cadaver skin was demonstrated. The extent of metabolism and the kind of metabolites differed depending on the skin source.

Conclusion

The skin as a drug metabolizing organ should not be underestimated, particularly in case of the formation of pharmacologically active compounds.

An amphiphilic cream containing mycophenolate mofetil might be a valuable contribution to the topical treatment of psoriasis and other inflammatory dermatoses. Topical use of mycophenolate mofetil in dermal therapy appeared to show essential advantages compared with the systemic administration (reduction of serious side effects) for the treatment of dermal diseases. Mycophenolate mofetil could be a promising alternative in the local treatment of psoriasis, but to confirm the results a clinical trial with psoriasis patients (chronic plaque type) would be necessary.

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