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Lipophilic metronidazole derivatives and their absorption through hairless mouse skin

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Previously we have shown that the diacyl glyceryl ester of naproxen is absorbed into excised mouse skin and slowly degraded to release naproxen. In the present work we have synthesised some organic acid and fatty acid derivatives of metronidazole, and studied the *in-vitro* degradation in aqueous buffer solutions and serum as well as their permeation through hairless mouse skin. The derivatives were enzymatically degraded in serum to form metronidazole. Only the acetic acid and butyric acid derivatives were able to permeate hairless mouse skin intact. The fatty acid derivatives released metronidazole within the skin. The metronidazole delivery through the skin was significant when the metronidazole oleate was used. This compound could therefore be considered as a suitable pro drug for dermal applications.

1. Introduction

In our previous work, we reported on the synthesis and testing of diacyl glyceryl esters of naproxen [1]. It was shown that these highly lipophilic pro drugs could be well absorbed into hairless mouse skin and would slowly release the drug by enzymatic hydrolysis. Other studies have shown that short chain alkyl ester prodrugs of naproxen can permeate human skin intact, and that flux of these prodrugs is similar to or lower than the flux of unmodified naproxen [2]. Naproxen is a good model compound to study different prodrug approaches, and the transdermal route could be a valuable alternative to oral administration. However, prodrugs releasing the drug in the skin would be most valuable as dermal drugs, and for such a use, naproxen has a rather limited value.

The antimicrobial drug metronidazole is used for topical application in the treatment of Acne rosacea and Acne vulgaris [3, 4]. This compound is very hydrophilic, and this attribute can be expected to limit its dermal absorption. Application of this drug in the form of a more lipophilic prodrug could therefore be advantageous, especially if the drug was released at a significant rate in the skin.

In the present work, we synthesised some aliphatic esters of metronidazole, with aliphatic side chains containing 1 to 17 carbon atoms. The effect of the length of the side chain on the hydrolysis in an aqueous buffer solution and serum and the absorption into hairless mouse skin and the release of free metronidazole were investigated.

2. Investigations, results and discussion

The metronidazole aliphatic ester derivatives were synthesised in a single step through carbodiimide coupling of the carboxylic acid to the alcohol group of metronidazole.

The pH rate profile for metronidazole laurate (MN-laurate) was obtained for aqueous buffer solutions at 60 °C. The prodrug was hydrolysed into metronidazole and a fatty acid moiety. MN-laurate was hydrolysed through specific base catalysis above pH 8 (Fig. 1). The H^+ concentration at low pH did not affect the hydrolysis rate. In alkaline solutions, the hydrolysis rate gradually decreased with the length of the aliphatic side chain. However, the longer side chain derivatives, MN-laurate, MN-palmitate and MN-oleate, hydrolysed about ten times faster than the short side chain derivatives, MN-acetate- and MN-butyrate in acidic solutions (Table 1). Both effects could be explained as a consequence of association of the longer aliphatic chains in aqueous solutions. The association would provide protection in alkaline solution, but some self-catalysis would be present in acidic solutions.

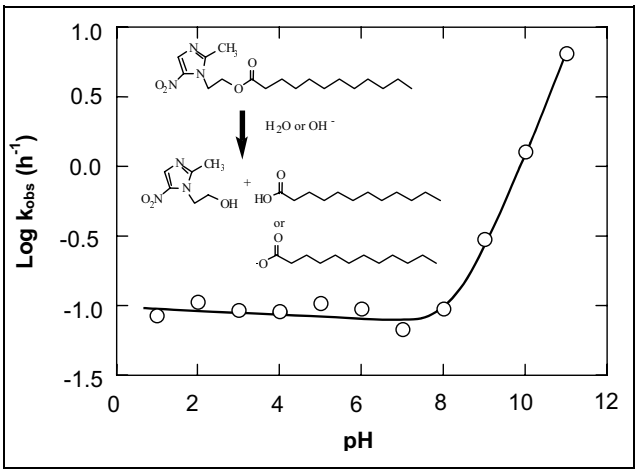

Fig. 1: pH-Degradation rate profile for metronitrazole laurate in KCl/HCl (pH 1) and citrate-phosphate-borate/HCl buffer (pH 2 to 11) at 60 °C. The hydrolysis reaction is shown

Metronidazole acetate was completely degraded into metronidazole and acetate in 4 h in serum at 37 °C (Fig. 2). The half-life ($t_{1/2}$) of this compound at 60 °C, in pH 9.0 aqueous buffer solutions, was about twice as long (49 min. vs. 68 min). This showed that the degradation in serum was mainly enzymatic. The half-life of MN-laurate was 8 times shorter (6.31 min) than that of MN acetate. This was in agreement with previous observations showing that longer side chain derivatives are better substrates for the serum esterase enzymes [1, 5].

The absorption through hairless mouse skin was measured, using propylene glycol to solvate the compounds in the donor phase (Table 2). Due to increased lipophilicity of the compound, a large increase in the absorption was observed when the acetate function was added to the me-

Table 1: Hydrolysis rate constants (k_{obs}) for the metronidazole (MN) derivatives

Compd.	pH 3	pH 9
	k_{obs} (h^{-1}) ± SD	k_{obs} (h^{-1}) ± SD
MN-acetate	0.0012 ± 0.0001	0.609 ± 0.03
MN-butyrate	0.0010 ± 0.0001	0.284 ± 0.06
MN-laurate	0.094 ± 0.001	0.301 ± 0.02
MN-palmitate	0.176 ± 0.097	0.106 ± 0.001
MN-oleate	0.166 ± 0.018	0.114 ± 0.01

Table 2: Flux and permeation of metronidazole and metronidazole derivatives through hairless mouse skin

Compound	Flux of compound $\mu g/(h \cdot cm^2) \pm SD$	Appearance of Metronidazole $\mu g/(h \cdot cm^2) \pm SD$	Total flux of compounds $nmol/(h \cdot cm^2) \pm SD$	Permeation of compounds* $(cm/h \pm SD) \times 10^6$
Metronidazole (MN)	0.17 ± 0.03	0.17 ± 0.03	1.0 ± 0.2	0.17 ± 0.03
MN-acetate	1.83 ± 1.46	1.63 ± 0.90	18.2 ± 12.2	3.9 ± 2.6
MN-butyrate	0.17	4.10 ± 0.93	24.8 ± 5.4	6.0 ± 1.3
MN-laurate	0	6.65 ± 6.13	39.1 ± 36.1	13.8 ± 12.7
MN-palmitate	0	1.58 ± 1.79	9.3 ± 10.5	3.8 ± 4.3
MN-oleate	0	3.80 ± 0.41	22.3 ± 2.4	9.7 ± 1.0

* Permeation of the compounds was calculated as the total flux of compounds (nmol h^{-1} cm^{-2}) divided by the concentration of the prodrug in the donor phase (nmol ml^{-1}).

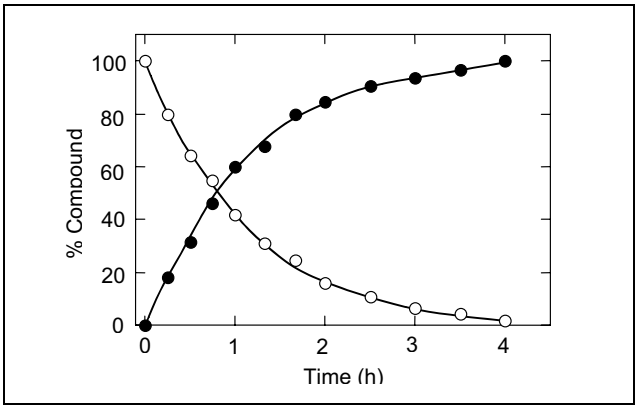

Fig. 2: Serum degradation of metronidazole acetate at 37 °C. The disappearance of metronidazole acetate (○) is accompanied by the quantitative appearance of metronidazole (●)

tronidazole molecule. About half of the MN-acetate appeared in the receptor phase as the unchanged compound, whereas the other half had been hydrolysed to give metronidazole. A previous study has shown that there is a significant permeation of some short chain metronidazole ester derivatives through excised human skin [6] within 72 h. Our study also showed a large increase in permeability of the skin after about 40 h incubation and a decrease in the release of free drug. However, excised skin is not viable for such an extended period [7], and the increase in permeability after 40 h was probably due to breakdown of the skin barrier function. In practice, we observed steady state flux of the compound and steady state release of metronidazole in the period between 5 and 35 h. The steady state flux data were used in the present study.

During this period, the butyric acid derivative could penetrate the skin only with difficulty, but the total flux, resulting mainly from the hydrolysis of the compound in the skin, was comparable to MN-acetate, approximately 4 $\mu g/cm^2$ h.

The total flux for metronidazole did not change significantly (taking the experimental error into account) from this value for MN-laurate and MN-oleate, whereas in the case of MN-palmitate, the flux was about half the flux of the other derivatives. Oleic acid can be used to enhance the permeation of hydrocortisone, whereas palmitic acid will have little effect [8]. Therefore, it is possible that oleic acid may facilitate its own absorption, whereas the high molecular weight of palmitate may reduce its absorption. The molar permeability of oleate was consistently higher than for the other compounds. Oleate derivatives of metronidazole can therefore be considered for dermal applications, especially since this compound is liquid at room temperature, and therefore does not have to be dissolved in cream vehicles.

3. Experimental

3.1. Materials

Metronidazole, 1-ethyl−3-(3-dimethylamonium propyl) carbodiimide (EDAC), lauric acid, hexanoic acid, palmitic acid, oleic acid and butyric anhydride were purchased from Sigma Chemical Co. (St. Louis, USA). Acetic anhydride, 4-dimethylaminopyridine (DMAP), Kieselgel 60 and TLC silica gel 60 F_{254} were obtained from Merck (Darmstadt, Germany). All other chemicals were of reagent grade.

3.2. Synthesis of metronidazole acetate

Metronidazole (1.0 g, 5.9 mmol) 1.1 g EDAC (5.9 mmol), 0.36 g acetic acid (5.9 mmol), and 0.1 g DMAP were dissolved in 40 ml Cl_2CH_2. After stirring for 4 h, the mixture was washed once with 5% $NaHCO_3$ and three times with H_2O. Subsequently, the solvent was partially removed in vacuo to afford 1 ml of solution. The product was then purified by Silica gel 60 column chromatography, using toluene and toluene/methanol (40/1 and 30/2) as eluent. Finally, the isolated compound was obtained in a 42% yield (533 mg): mp 70–71 °C; ^{1}H NMR ($CDCl_3$): δ 7.96 (s, 1 H), 4.59 (t, J = 5 Hz, 2 H), 4.41 (t, J = 5 Hz, 2 H), 2.52 (s, 3 H), 2.02 (s, 3 H).

3.3. Synthesis of metronidazole laurate

Metronidazole (1.02 g, 6 mmol), 1.2 g EDAC (6 mmol), 1.2 g lauric acid (5.9 mmol) and 0.12 g DMAP were dissolved in 40 ml Cl_2CH_2. After stirring for 3 h, the mixture was washed once with 5% NaH_2CO_3 and three times with H_2O. Subsequently the solvent was partially removed in vacuo to afford 1 ml of solution. The product was then purified by Silica gel 60 column chromatography, using toluene and toluene/methanol (40/1 and 30/2) as eluent. Finally, the isolated compound was obtained in a 36% yield (758 mg): m.p. 35–36 °C; ^{1}H NMR ($CDCl_3$): δ 7.98 (s, 1 H), 4.61 (t, J = 5 Hz, 2 H), 4.42 (t, J = 5 Hz, 2 H), 2.56 (s, 3 H), 2.25 (t, J = 7 Hz, 2 H), 1.50 to 1.65 (m, J = 7 Hz, 2 H), 1.25 (m, 16 H), 0.88 (t, 3 H)

3.4. Synthesis of metronidazole butyrate

Metronidazole (1.02 g, 6 mmol), 1 g butyric anhydride (6.3 mmol) and 0.12 g DMAP were dissolved in 40 ml Cl_2CH_2. After stirring for 12 h, the mixture was washed once with 5% $NaHCO_3$ and three times with H_2O. Subsequently, the solvent was partially removed in vacuo to afford 1 ml of solution. The product was then purified by Silica gel 60 column chromatography, using toluene and toluene/methanol (40/1 and 30/2) as eluent. Finally, the isolated compound was obtained in a 52% yield (754 mg): m.p. 37–38 °C; ^{1}H NMR ($CDCl_3$): δ 7.95 (s, 1 H), 4.58 (t, 2 H), 4.40 (t, 2 H), 2.51 (s, 3 H), 2.31–2.20 (m, 2 H), 1.62–1.54 (m, 2 H), 0.99–0.86 (m, 3 H)

3.5. Synthesis of metronidazole palmitate

Metronidazole (1 g, 5.9 mmol), 1.2 g EDAC (6.2 mmol), 1.5 g palmitic acid (5.9 mmol) and 0.12 g DMAP were dissolved in 40 ml Cl_2CH_2. After stirring for 3 h, the mixture was washed once with 5% $NaHCO_3$ and three times with H_2O. Subsequently, the solvent was partially removed in vacuo to afford 1 ml of solution. The product was then purified by Silica gel 60 column chromatography, using toluene and toluene/methanol (40/1 and 30/2) as eluent. The compound was recrystallised from methanol to give white crystals in a 58% yield: m.p. 51–52 °C; ^{1}H NMR ($CDCl_3$): δ 7.97 (s, 1 H), 4.59 (t, J = 5 Hz, 2 H), 4.41 (t, J = 5 Hz, 2 H), 2.52 (s, 3 H), 2.25 (t, J = 7 Hz, 2 H), 1.61–1.53 (m, 2 H), 1.25 (m, 24 H), 0.88 (t, J = 5 Hz, 3 H)

3.6. Synthesis of metronidazole oleate

Metronidazole (1 g, 5.9 mmol), 1.2 g EDAC (6.2 mmol), 1.7 g oleic acid (5.9 mmol) and 0.12 g DMAP were dissolved in 40 ml Cl_2CH_2. After stirring for 4 hours, the mixture was washed once with 5% $NaHCO_3$ and three times with H_2O. Subsequently, the solvent was partially removed in vacuo, leaving an oily residue. The oily product was then purified by Sili-

ca gel 60 column chromatography, using toluene and toluene/methanol (40/1 and 30/2) as eluent. The purification produced a light yellow oil with a 72% yield (1869 mg); ^{1}H NMR ($CDCl_3$): δ 7.96 (s, 1 H), 5.36 to 5.31 (m, 2 H), 4.59 (t, 2 J = 5 Hz, H), 4.40 (t, J = 5 Hz, 2 H), 2.52 (s, 3 H), 2.25 (t, J = 7 Hz, 2 H), 2.01–1.96 (m, 4 H), 1.58–1.52 (m, 2 H), 1.26 (m, 20 H), 0.87 (t, J = 5 Hz, 3 H).

3.7. Degradation studies

For pH 1 solution, a KCl/HCl buffer was used. For solutions in the pH range of 2 to 11, a citrate-phosphate-borate/HCl buffer was used [9]. Stock solution (1 mg/ml) was made in ethanol. Fifteen or 50 μl of the drug stock solution were added to 1.5 ml a buffer solution, which was kept at 60 ± 0.1 °C in a temperature-controlled sample rack in an AS-4000 (Merck-Hitachi) autosampler. The changes in the drug concentration with time were monitored by HPLC. The HPLC system consisted of a Constametric 3000 (Milton Roy) solvent delivery system with a SP8450 (Spectra-Physics) variable wavelength detector, using a 150 mm, 4.6 mm i.d., 5 μm bead, C18 reverse-phase column. The detection wavelength was 320 nm. The serum degradation rate studies were done by adding 0.1 ml of a 3 mg/ml methanol stock solution to 2.9 ml human serum and incubating at 37 °C. Samples (0.1 ml) were drawn at predetermined intervals and 0.1 ml $HClO_4$ and 0.8 ml of CH_3CN added to precipitate the protein. After centrifugation, supernatant was filtrated, and the concentration of the metronidazole derivative was analysed by HPLC. The mobile phases and retention times for the different drugs were as follows: for MN-acetate: acetonitrile, acetic acid, water (39 : 1 : 5 : 60), 2.5 min; for MN-butyrate: acetonitrile, acetic acid, water (49 : 1 : 50), 3.5 min; for MN-laurate: acetic acid, acetonitrile (1 : 99) 3.4 min; for MN-palmitate and oleate: acetic acid, 2-propanol, acetonitrile (1 : 20 : 79), with a 3.5-min retention time for the palmitate derivative and 4.2 min for the oleate derivative.
The first-order degradation rate constant, k_{obs}, was obtained from linear regression of the peak heights vs. time.

3.8. Permeation studies

Female hairless mice (3CH/Tif hr/hr), obtained from Bommice (Denmark), were sacrificed by cervical dislocation, and their full-thickness skins were removed. The outer surface of the skin was rinsed with 35% (v/v) CH_3OH in H_2O and subsequently with distilled H_2O to remove any contamination. The skin was placed in Franz diffusion cells of type FDC 400 15 FF (Vangard International Inc., USA). The receiver compartment had a volume of 12.3 ml. The surface area of the skin in the diffusion cell was 1.77 cm^2. The receptor phase consisted of phosphate buffer saline pH 7.4 (Ph.Eur., 2nd Ed., VII.1.3.), containing 0.3% (w/v) Brij-58 to ensure sufficient drug solubility in the receptor phase. The receptor phase was sonicated under vacuum prior to usage to remove dissolved air. The skin diffusion cells were stirred with a magnetic bar and kept at 37 °C by circulating H_2O through an external jacket. The donor phase consisted of 1 mg/ml of the prodrug in propylene glycol. Two ml of the donor phase were applied to the skin surface, and the donor chamber was covered with Parafilm®. Samples (100 μl) of receptor phase were removed from the cells at various time intervals of up to 48 h and replaced with a fresh buffer solution. The samples were kept frozen until analysed by HPLC.

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