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In vitro evaluation of dermal prodrug delivery — transport and bioconversion of a series of aliphatic esters of metronidazole

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Summary

The ability of a series of aliphatic ester prodrugs to improve cutaneous delivery of metronidazole has been investigated. In permeation studies using full thickness human skin in vitro the derivatives lead only to a 1.5-fold enhancement of permeation compared to the parent drug. The influence of non-specific cutaneous hydrolytic enzymes on the rate of hydrolysis of the ester derivatives was determined in homogenates prepared from human skin. It was observed that the susceptibility of the esters to undergo enzyme-catalyzed cleavage was strongly affected by the length of the acyl side chain making the valerate and caproate esters the best substrates. The experimental data demonstrate that no single ester derivative can be selected with optimal properties regarding both skin permeation and enzymatic regeneration to metronidazole. Due to the relatively small variation in permeation rate of the compounds, however, the butyrate ester seems to fulfill the requirements of good permeation characteristics as well as a quick conversion to metronidazole ($t_{1/2}$ in skin homogenate is 1.0 h at 37°C).

Introduction

A rational way to improve the efficiency of drugs for the dermal route would be to manipulate the physicochemical properties of the drugs by selecting drug derivatives with the potential for increasing the rate of diffusion through the skin barrier. A promising approach in this respect is development of prodrugs which after diffusion into or through the skin undergo reversion to the active drug.

Thus the prodrug approach in dermal drug delivery has been the subject of several, recent investigations and various drugs have been considered, e.g. vidarabine (Yu et al., 1979), aspirin (Loftsson and Bodor, 1981), theophylline (Sloan and Bodor, 1982; Sloan et al., 1984a), 6-thiopurines (Sloan et al., 1983), 5-fluorouracil (Møllgaard et al., 1982; Sloan et al., 1984a), metronidazole (Bundgaard et al., 1983), indomethacin (Sloan et al., 1984b), dithranol (Wiegrebe et al., 1984) and mitomycin C (Mukai et al., 1985; Hashida et al., 1985). These investigations show that although the effect generally is relatively small, drug derivatives can be made to permeate the skin more readily

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than the parent compounds. However, guidelines for enabling optimal dermal delivery by use of the prodrug approach are still lacking. Few reports on prodrugs have dealt with derivatives consisting of homologous series where systematic evaluation of their physicochemical properties feasible for penetration across human skin have been performed.

Therapeutic activity demands reversion of the prodrug to the active agent accomplished by enzymatic or non-enzymatic reactions. Many of the prodrugs studied so far possess an ester linkage capable of undergoing enzyme-catalyzed cleavage to the parent drug. It is well documented that a number of metabolic processes proceeds by dermal enzymes (for reviews, see Täuber, 1982; Pannatier et al., 1978; Bucks, 1984), and Pannatier et al. (1981a) have studied the enzymatic hydrolysis of a series of aliphatic esters of *p*-nitrobenzoic acid in mouse skin homogenate. To our knowledge, however, a systematic study pertinent to the influence of variation in the acyl side chain of the ester portion on the rate of human skin enzyme-catalyzed hydrolysis has not been performed.

Using a series of aliphatic esters of metronidazole the present study was undertaken with the aim of elucidating the effect of variation of the ester acyl chain length on human skin permeation as well as sensitivity to undergo attack by human skin hydrolases. Presently similar experiments are being carried out with aromatic and amino acid esters of metronidazole. Together these systematic studies are intended to provide more general information about the potential utility of ester type prodrugs for dermal drug delivery.

Materials and Methods

Apparatus

HPLC analysis was carried out using a Waters Associates Model 6000 A constant-flow pump equipped with a Pye Unicam PU 4020 variable wavelength detector and a Rheodyne Model 7125 injection valve with a 20 μl loop. The column used, 250 × 4 mm, was packed with LiCrosorb RP-8 (7 μm particles) and equipped with a guard column. Readings of pH were done with a Radiometer Type pH M 26 meter at the temperature of study. Homogenization of the skin specimens was carried out using a Mikro-dismembrator, model B. Braun equipped with a 7 ml teflon chamber and a chrome steel sphere with a diameter of 10 mm.

Chemicals

Metronidazole was obtained from Dumex A/S, Copenhagen. The acetate, propionate, butyrate, valerate and caproate esters of metronidazole (Table 2) were synthesized according to a previous study (Johansen and Larsen, 1985). The solvent used in the HPLC procedures was of chromatographic grade. All other chemicals and buffer substances were of reagent grade.

Analysis of metronidazole and the ester derivatives

The investigated compounds were analyzed by HPLC methods according to previously described procedures (Bundgaard et al., 1983; Johansen and Larsen, 1985).

Measurements of partition coefficients

The partition coefficients of the ester derivatives were determined in *n*-octanol/0.05 M phosphate buffer pH 7.4 at ambient temperature. The solute concentrations in the aqueous phase, before and after partition, were determined by HPLC.

Permeation studies using excised human skin

The experimental procedures for the permeation studies were as described previously (Møllgaard and Hoelgaard, 1983a). In brief, the excised human mamma skin was mounted in open diffusion cells with a diffusion area of 1.8 cm^2. A 0.05 M isotonic phosphate buffer solution of pH 7.4 containing 0.01% of mercury (II) chloride as a preservative and 0.5% of Pluronic F68 as solubilizer was used as receptor phase (7.5 ml). Test solutions of the compounds were made in ethanol containing 18% w/v of propylene glycol. An aliquot (56 μl) of this solution corresponding to 1 μmol test compound per cm^2 skin was applied to the skin samples. The ethanol evaporated from the skin surface within a few minutes. At appropriate intervals samples of 2 ml were removed from the receptor compartment and replaced with fresh

receptor phase. The samples were analyzed by HPLC. In the permeation studies of the ester compounds the receptor phase was analyzed for metronidazole as well as for intact ester. The permeation studies were carried out in quadruplicate.

Preparation of human skin homogenate

Approximately 300 mg of skin specimens (3×3 mm^2) were placed in a teflon chamber containing a small chrome steel sphere. After freezing the skin ($\sim -200°C$ by exposing the chamber to liquid nitrogen for 1–2 min); the chamber was placed in the Mikro-dismembrator apparatus and vigorously shaken for 30 s. This method produced a fine yellowish-white powder which by freeze drying lost approximately 65% in weight. After lyophilization the powder was suspended in 0.05 M phosphate buffer, pH 7.4, in a concentration corresponding to 5% w/v dry skin. After incubation at 5°C for 24 h the suspension was centrifuged for 5 min at $10,000 \times g$ and the supernatant containing enzymes was removed and stored at $-18°C$.

Determination of the enzyme activity in human skin homogenates

The supernatant was added to a phosphate buffer pH 7.4 at 37°C. After equilibration for 5 min 500 µl of an aqueous solution of the individual metronidazole ester were added making a final concentration in the range 0.015–0.03 µmol/ml. At appropriate intervals 500 µl samples were withdrawn and deproteinized with 1500 µl methanol. The mixture was vortexed for 30 s, centrifuged for 2 min at $10,000 \times g$ and analyzed for intact ester.

Results and Discussion

Enzymatic hydrolysis of metronidazole esters

Various in vitro methods have been employed in skin metabolism studies including skin fragments or homogenates (e.g. Yu et al., 1980; Pannatier et al., 1981b and Anderson et al., 1982). These studies have generally been conducted using skin from various animal species. Realizing the possible differences that exist in the cutaneous enzyme systems of animals and man, the use of human skin is preferable in assessing bioconversion of prodrugs for dermal delivery.

Skin homogenate was used in order to liberate both intra- and extracellular enzymes. The tenacious and elastic nature of the outermost layer of the skin represents problems in most homogenizers. Therefore, the method of generating the skin homogenates has to be considered carefully. The procedure in this study includes an effective grinding of the frozen tissue in a ball mill and at the same time prevents the skin from exposure to fluid medium and elevated temperatures. The obtained powder was inspected microscopically to ensure that the integrity of cells was at least partly lost.

By incubation of lyophilized skin powder with phosphate buffer for different periods of time it was found that the supernatants exhibited maximal enzyme activity (using metronidazole butyrate as the substrate) already after an incubation period of 22 h. This activity was found to remain at the same level in supernatants derived from incubation periods of up to 4 days. Täuber (1982) has described that the physiological microbial flora may contribute to the enzymatic activity. However, microorganisms do not seem to influence the enzymatic activity towards the esters of metronidazole as indicated by the invariability of the enzymatic activity after the equilibration period. This observation is in accordance with the result of Bundgaard et al. (1983) who found that the enzyme activity in the receptor phase due to leaching of enzymes from the dermal site of human skin remained constant at least for 70 h.

The rates of hydrolysis of the aliphatic esters of metronidazole were determined at various substrate concentrations. The esters were completely converted to metronidazole and the hydrolysis was found to follow strict first-order kinetics indicating that the enzymes were present in excess. The influence of the substrate concentration on the observed pseudo-first-order rate constant for hydrolysis of metronidazole butyrate is shown in Fig. 1. As seen the degradation rate increases with decreasing substrate concentration due to a larger excess of esterases. Consequently, kinetic studies

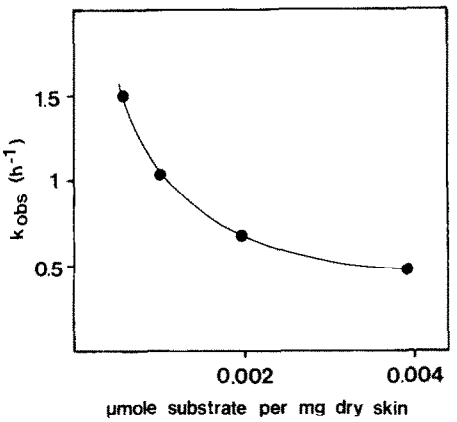


Fig. 1. Influence of the substrate concentration on the observed pseudo-first-order rate constant for hydrolysis of metronidazole butyrate to metronidazole in human skin homogenate (pH 7.4, 37°C)

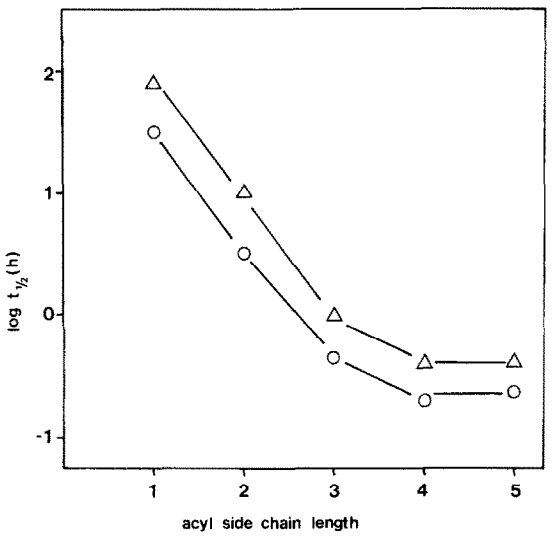


Fig. 2. Semilogarithmic plot of $T_{1/2}$ (h) of hydrolysis of metronidazole esters to metronidazole in human skin homogenate (△) and plasma (○) (the data used are from Table 2).

were performed at two different substrate concentrations allowing k_{obs} to be derived at a specific value of substrate concentration (2.0×10^{-3} μmol/mg dry skin) by interpolation.

As a result of the complex nature of human skin a pH gradient across the skin layers exists. Thus the pH of the outermost layers is 4.5–5.5 while in dermis the pH is about 7 (Katz and Poulsen, 1971). The influence of pH of the substrate medium on the rate of enzymic-catalyzed reversion of the butyrate ester to metronidazole is presented in Table 1. It appears that the hydrolysis rate in the skin homogenates is largest at physiological pH 7.4 and decreases at lower pH-values. A substrate medium at pH 7.4 was therefore chosen for further studies.

TABLE 1

PSEUDO-FIRST-ORDER RATE CONSTANTS FOR DEGRADATION OF METRONIDAZOLE BUTYRATE IN SKIN HOMOGENATE AT DIFFERENT pH-VALUES AT 37°C

Buffer	pH	k_{obs} (h^{-1}) [a]
0.05 M borate	8.50	0.238
0.05 M phosphate	7.40	0.639
0.05 M phosphate	6.00	0.302
0.05 M acetate	5.00	0.191
0.05 M acetate	4.00	0.0150

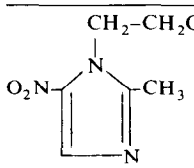
[a] Substrate concentration: 0.002 μmol/mg dry skin.

In Table 2 are listed the half-lives for hydrolysis of the esters in the presence of 0.91% w/v skin homogenate. Previously reported results for the cleavage of the aliphatic esters of metronidazole in buffer solution and in human plasma (Johansen and Larsen, 1985) are included for comparison. From Table 2 it is obvious that the rate of hydrolysis of the esters is markedly increased in the presence of human skin hydrolases, the effect being most pronounced for the valerate and caproate esters. The rate increase for the latter esters amounts to a factor of approximately 2000. This observed tendency of enhanced rate of bioconversion with increased length of the acyl side chain parallels the findings of Pannatier et al. (1981a) who found, in a series of linear aliphatic esters of *p*-nitrobenzoic acid, increasing susceptibility of the ester substrates to undergo hydrolysis by elongation of the chain length from the methyl to the butyl ester.

The rate data from the hydrolysis of the ester derivatives in skin homogenate and human plasma are also illustrated in Fig. 2, and it is apparent that the sensitivity to enzyme-mediated hydrolysis of the esters in skin homogenate and in plasma is

TABLE 2

$T_{1/2}$-VALUES OF HYDROLYSIS OF METRONIDAZOLE ESTERS TO METRONIDAZOLE IN HUMAN SKIN HOMOGENATE, PLASMA AND BUFFER SOLUTION



R	Compound	$t_{1/2}$(h) at 37°C			
		I 0.05 M phosphate buffer pH 7.40	II 2.5% human plasma [a]	III 0.91% w/v homogenate [a] (dry weight basis)	I/III ratio
-H	Metronidazole				
-C(=O)-CH_3	Metronidazole acetate	400	32	74	5.4
-C(=O)-CH_2CH_3	Metronidazole propionate	507	3.2	11	46
-C(=O)-$CH_2CH_2CH_3$	Metronidazole butyrate	890	0.45	1.0	890
-C(=O)-$CH_2CH_2CH_2CH_3$	Metronidazole valerate	802	0.21	0.4	2010
-C(=O)-$CH_2CH_2CH_2CH_2CH_3$	Metronidazole caproate	862	0.24	0.4	2160

[a] In 0.05 M phosphate buffer at pH 7.4.

almost similar. These results suggest the possibility of using human plasma to assess the hydrolysis rates of linear aliphatic ester prodrugs for dermal delivery. However, preliminary experiments in this laboratory imply that the above-mentioned relationship cannot be expanded to encompass amino acid esters of metronidazole.

Permeation rate

Propylene glycol was chosen as the vehicle since its use has been well defined in this laboratory. The test solutions of the various esters and metronidazole were made with the same molar drug concentration allowing for comparison of the same dose of drug, but the variability of thermodynamic activity (degree of saturation) was not taken into account. However, previous studies in our laboratory have shown that the thermodynamic activity of the drug in propylene glycol is not important as this vehicle leaves the skin surface by permeation simultaneously with the drug (Møllgaard and Hoelgaard, 1983b). Fig. 3 represents a typical plot of data for permeation of metronidazole and its valerate and propionate esters. The shape of the profiles of the three other esters (acetate, butyrate and caproate) was very much alike. A lag-time was observed representing the time required for the drug to reach the receptor phase. Steady-state diffusion was not established as it requires a constant drug concentration at the skin surface. The permeation was almost terminated after 72 h for all six compounds reaching a plateau of 63–97% of dose transported across the skin. As metronidazole is a relatively good penetrant, the esters show only a minor improvement of the transport rate.

In the permeation experiments with metronidazole propionate, butyrate, valerate and caproate,

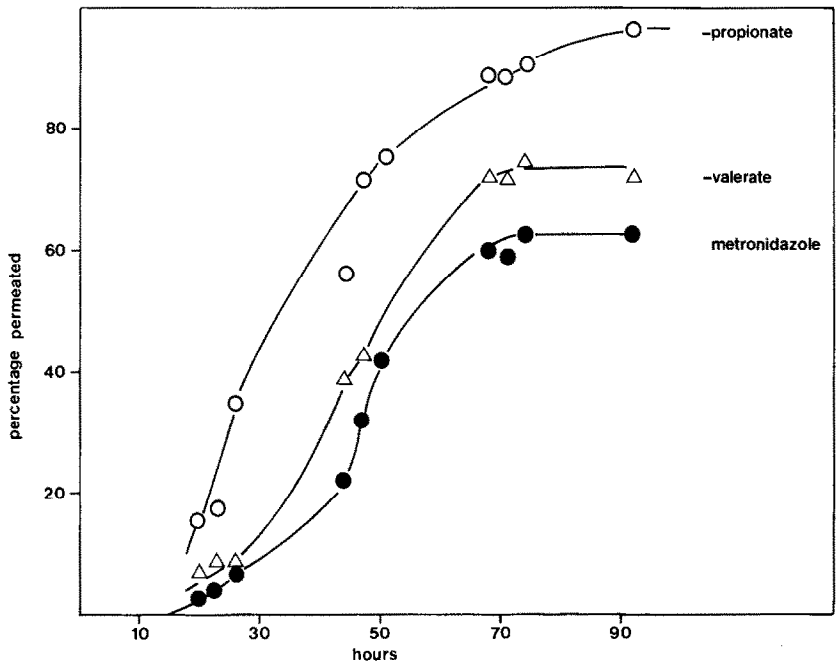


Fig. 3. Permeation of metronidazole (●), metronidazole propionate (○) and metronidazole valerate (△) through human skin in vitro. The data for the propionate and the valerate esters represent free metronidazole. Applied amount per cm^2: about 1 μmol test compound and 10 mg propylene glycol in an ethanolic solution.

no unchanged ester was detected in the receptor phase during the study (detection limit 20 ng · ml^{-1}). The inability to detect these esters may be due to loss by hydrolysis during the diffusion across the skin and/or hydrolysis in the receptor phase due to leached enzymes from the dermis side. A leaching of hydrolytic enzymes takes place rapidly during a permeation experiment using excised human skin. It is essentially complete after a period of about 20 h and the enzyme activity in the receptor phase remains constant for at least an additional 70 h (Bundgaard et al., 1983). For comparison the degradation of metronidazole butyrate was measured in receptor phase exposed to intact human skin for 24 h. $T_{1/2}$ was found to be 13.6 h, i.e. hydrolysis is about 13 times slower than in the homogenate ($T_{1/2} = 1$ h). Although these data do not allow a strict analysis to be made of the relative magnitude of the two ways of ester hydrolysis, during diffusion in the skin or in the receptor phase, it appears reasonable to conclude that enzymatic degradation takes place predominantly within the skin simultaneously with the permeation.

In the permeation experiment with metronidazole acetate intact ester was detected in the

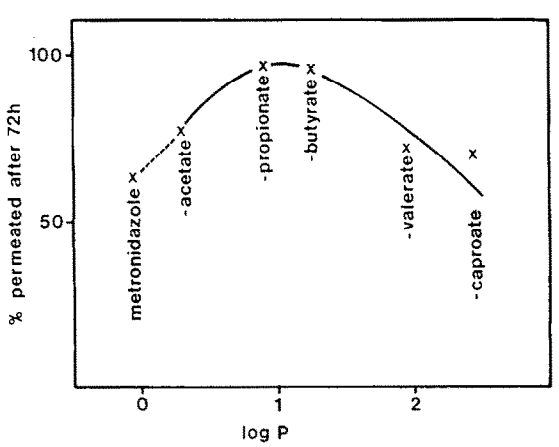


Fig. 4. 72 h permeation of metronidazole and its aliphatic esters through human skin in vitro versus their partition coefficients (*n*-octanol/buffer pH 7.4).

receptor phase. This is in good agreement with the fact that metronidazole acetate is the most stable ester in the skin homogenate ($T_{1/2} = 74$ h). The relationship between the permeation rate (percent permeated after 72 h) and the drug partition coefficient *n*-octanol/buffer for all the esters is shown in Fig. 4. Although the permeation rate of the six substances only exhibits minor differences, the trend is a parabolic relationship with a maximum at log P ~ 1. Such a parabolic relationship revealing an optimal partition coefficient is in agreement with the nature of the horny layer as a lipid–protein–water matrix. As molecular modification produces a more hydrophobic compound, aqueous solubility decreases, and the partition coefficient and solubility would tend to cancel one another in terms of increased permeation.

Conclusion

The aim of topical therapy is mainly to provide a high drug concentration within the skin. The success of prodrug approach in this respect is based upon improved permeation of the skin and rapid reversion to the parent drug. The barrier function of human skin is confined to stratum corneum while the viable epidermis is regarded as the main metabolic layer. The data generated in this study show that molecular modifications in a homologous series of aliphatic esters of metronidazole influence the regeneration rate to the parent drug as well as the permeation rate. The hydrolysis of the metronidazole esters was found to be markedly enhanced by the presence of enzymes from human skin homogenate. The most efficient enhancement was observed for the derivatives with the longest carbon chains, where the increment was approximately 2000-fold. With increasing lipophilicity of the substrate, corresponding to an elongation of the acyl chain, the enzymes in human skin mediate hydrolytic cleavage more readily.

Derivatizing metronidazole to aliphatic esters only influences its permeation to a small extent. However, a parabolic relationship identifies a specific optimal partition coefficient for these esters.

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