International Journal of Pharmaceutics, 15 (1983) 285–292
Elsevier

Leaching of hydrolytic enzymes from human skin in cutaneous permeation studies as determined with metronidazole and 5-fluorouracil pro-drugs

Hans Bundgaard, Annie Hoelgaard and Birgitte Møllgaard

The Royal Danish School of Pharmacy, Departments of Pharmaceutical Chemistry AD and Pharmaceutics, 2 Universitetsparken, DK-2100 Copenhagen (Denmark)

(Received October 30th, 1982)
(Accepted December 21st, 1982)

Summary

The leaching of hydrolytic enzymes out of the dermal side of excised human skin into the receptor phase of a permeation cell was studied using the esters metronidazole benzoate and 1-butyryloxymethyl-5-fluorouracil as test compounds. The enzyme activity in the receptor phase increased rapidly and was essentially complete after an exposure time of 20 h. The half-life for the hydrolysis of metronidazole benzoate in the phosphate buffer, pH 7.4, receptor phase exposed to the skin for 20 h was 3.1 h, whereas that in non-exposed receptor phase was 1000 h. It is emphasized that receptor phase metabolism due to leached enzymes may be of significance when assessing concurrent transport and metabolism of drugs or pro-drugs in permeation studies using human skin.

Metronidazole benzoate permeated more readily through the skin than metronidazole which was ascribed to its higher skin–vehicle partition coefficient. Only hydrolyzed metronidazole was detected in the receptor phase.

Introduction

Recent studies using hairless mouse skin preparations have shown that enzymes may leach out from the dermis side of skin into the receptor phase during permeation studies. Ando et al. (1977) and Yu et al. (1979) showed that adenosine deaminase, an enzyme that metabolizes the antiviral drug, vidarabine, into 9-β-D-arabinofuranosylhypoxanthine, rapidly leaches out, the leaching being complete after a period of about 6 h. Yu et al. (1980) also found that esterase enzymes capable

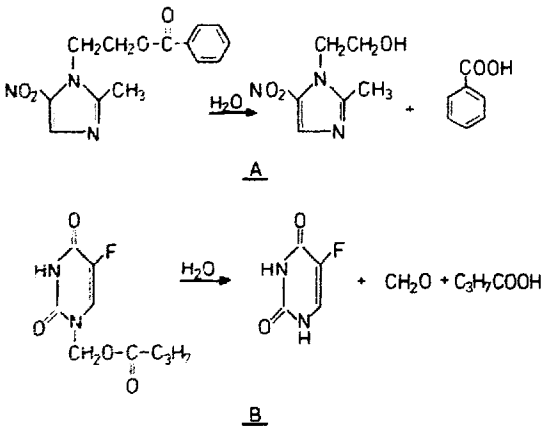

Fig. 1. Hydrolysis of metronidazole benzoate (A) and 1-butyryloxymethyl-5-fluorouracil (B).

of hydrolyzing the 3'-valerate ester of vidarabine leach out from ther dermis side of a hairless mouse skin preparation and that the leaching was essentially completed within 2 h.

A previous study (Møllgaard et al., 1982) indicated that enzymes capable of hydrolyzing N-1-acyloxymethyl derivatives of 5-fluorouracil also leach out from the dermis side of a whole human skin preparation during a permeation study with the skin samples mounted in open diffusion cells. Since knowledge of receptor phase drug metabolism due to leached enzymes may be of importance when studying concurrent permeation and metabolism of drugs or pro-drugs in the skin mounted in permeation cells, it was desirable to determine the rate at which enzymes were leaching out of the human skin preparations into the receptor phase compartment.

To this end the rates of hydrolysis of the esters metronidazole benzoate and 1-butyryloxymethyl-5-fluorouracil (Fig. 1) were determined in receptor phase solutions which had been exposed to the human skin preparations for various times. In addition, a comparative permeation study has been made of metronidazole and its benzoate ester, the results obtained being discussed in view of the enzyme-leaching experiments.

Materials and Methods

Apparatus

Ultraviolet spectral measurements were performed with a Shimadzu UV-190 spectrophotometer, using 1 cm cuvettes. Readings of pH were carried out on a Radiometer Type PHM 26 meter at the temperature of study. High-performance liquid chromatography (HPLC) was done with a Spectra-Physics Model 3500B instrument equipped with a 10 μl loop injection valve and a variable wavelength UV-detector. The column used, 250 × 4 mm, was packed with LiChrosorb RP-8 (7 μm particles) (E. Merck, Darmstadt, F.R.G.) and equipped with a guard column.

Chemicals

Metronidazole and metronidazole benzoate were kindly provided by Dumex A/S, Copenhagen. 1-Butyryloxymethyl-5-fluorouracil was from a previous study (Johansen et al., 1983). Buffer substances and all other chemicals or solvents used were of reagent grade.

Analysis of metronidazole and its benzoate ester

An HPLC method was used to analyze the receptor phase samples or aqueous buffer samples for metronidazole and its benzoate ester. For analysis of metronidazole the reversed-phase column was eluted isocratically at ambient temperature with a mobile phase consisting of 0.005 M acetate buffer, pH 4.5–methanol (6 : 4 v/v). The flow rate was 1.6 ml · min^{-1} and the column effluent was monitored at 317 nm. Under these conditions metronidazole showed a retention time of 2.3 min whereas the benzoate ester appeared as a broad peak after 12 min.

The chromatographic determination of the benzoate ester was done using a mobile phase of 0.005 M acetate buffer pH 4.5–methanol (35 : 65 v/v), the other chromatographic conditions being the same as described above. The ester showed a retention time of 2.8 min whereas metronidazole eluted with the solvent front. Quantitation of the compounds was done by measuring the peak heights in relation to those of standards (prepared in water with 5% ethanol) chromatographed under the same conditions. The detection limit was about 0.05 μg · ml^{-1}.

It was confirmed that substances leached from the skin samples into the receptor phase showed no interferences with the peaks of the two compounds.

Hydrolysis of metronidazole benzoate

The rate of hydrolysis of the benzoate ester was determined in 0.05 M phosphate buffer solution, pH 7.40 (pure receptor phase), the buffer containing 80% human plasma as well as in receptor phase samples which had been exposed to skin preparations for various times, using the HPLC procedures described above.

For the runs with the receptor phase samples 100 μl of an ethanolic solution of metronidazole benzoate were added to 3 ml sample portions to give an initial ester concentration of about 0.07 mg · ml^{-1}. The solutions were kept at 37°C in a water-bath and at suitable intervals 10-μl aliquots were injected onto the column and analyzed for remaining ester (in some runs also for free metronidazole). Pseudo-first-order rate constants for the hydrolysis were obtained from linear plots of the logarithm of remaining ester against time.

In the hydrolysis of metronidazole benzoate in 0.05 M phosphate buffer, pH 7.4, containing 80% human plasma, 200 μl plasma solution samples were taken at suitable intervals and immediately deproteinized by mixing with 1000 μl of ethanol. After centrifugation for 2 min, 10 μl of the clear supernatant was injected on HPLC and analyzed for remaining ester. First-order rate constants were derived as described above.

Due to the very slow rate of hydrolysis of the ester in pure phosphate buffer, the hydrolysis kinetics in this case was determined by the initial rate method. An accurately weighed sample of metronidazole benzoate (about 5 mg) was dissolved in

0.50 ml of ethanol and then added to 20 ml of buffer solution. The resulting solution was kept at 37°C and at appropriate intervals aliquots were removed and analyzed for metronidazole. The initial rate of metronidazole formation was determined from the slope of a linear plot of amount of metronidazole released vs time. Dividing this rate by the initial concentration of ester afforded the pseudo-first-order rate constant for the hydrolysis. The rate constants obtained using two different initial ester concentrations agreed within 3%.

Hydrolysis of 1-butyryloxymethyl-5-fluorouracil

The rate of hydrolysis of this compound to 5-fluorouracil in receptor phase samples which had been exposed to skin preparations for various times was determined using a HPLC procedure as described previously (Møllgaard et al., 1982).

Measurement of solubility and partition coefficients

The solubility of metronidazole and the benzoate ester in water and propylene glycol was determined at 22°C by rotating excess amounts in the appropriate solvent for 48 h. The concentrations of the compounds in their saturated solutions were determined by HPLC. The partition coefficients were determined in octanol/water. The solute concentrations in the aqueous phase was determined spectrophotometrically at 317 nm before and after partitioning.

Permeation–metabolism studies using excised human skin

The experimental procedures for the permeation studies were essentially as described previously (Møllgaard et al., 1982). The skin was stored at −18°C for 4 days after removal under autopsy from a single donor and was allowed to thaw gradually at room temperature just before use. The excised human skin mounted in open diffusion cells had an available 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 was used as receptor medium (7.5 ml). Solutions of the compounds were made in ethanol containing 20% of propylene glycol. An aliquot (50 μl) of this solution (corresponding to 175 μg of the test compound) was applied to the skin samples per cm^2. The ethanol evaporated within a few minutes. At appropriate intervals samples of 2 ml were removed from the receptor phase, replaced with fresh buffer and analyzed as described above. The permeation data given for each compound are the mean of 4 determinations.

Leaching of enzymes from dermis side

Five pieces of whole abdominal human skin free from subcutaneous fat were mounted in open diffusion cells as in the permeation study. The receptor phase (7.5 ml of isotonic 0.05 M phosphate buffer solution of pH 7.4) was stirred at 37°C. At various times the receptor phases were removed and stored at −20°C until they were incubated with metronidazole or 1-butyryloxymethyl-5-fluorouracil to determine the enzyme activity on the basis of measurement of rates of hydrolysis of the esters.

Results and Discussion

Leaching of enzymes

The rates of hydrolysis of metronidazole benzoate and 1-butyryloxymethyl-5-fluorouracil were determined at 37°C in receptor phase solutions exposed to the human skin samples in the diffusion cells for various times. For both compounds the hydrolysis was found to follow strict first-order kinetics as demonstrated for metronidazole benzoate in Fig. 2. The data in Figs. 3 and 4, which are plots of the observed pseudo-first-order rate constants for the hydrolysis occurring in the receptor phase vs the leaching times, reveal that the leaching of esterase enzymes is essentially complete after a period of about 20 h and that the enzyme activity in the receptor phase remains constant for at least an additional 50 h. It is of interest to note that the enzyme activity vs leaching time curves for metronidazole benzoate and the N-acyloxymethyl derivative of 5-fluorouracil are qualitatively quite similar, indicating that the same enzymes are involved in the hydrolysis of these esters.

The results obtained show that leaching of esterase enzymes takes place rapidly during permeation studies using excised human skin preparations and that receptor phase drug metabolism due to leached enzymes may be of great importance in evaluating permeation studies of hydrolyzable drugs or pro-drugs such as esters. Thus, whereas the half-life for the hydrolysis of metronidazole benzoate in 0.05 M phosphate buffer at 37°C was found to be 1000 h the half-life was only 3.1 h in the same buffer exposed to the skin preparation for 20 h. For the 5-fluorouracil derivative the corresponding half-lives are 114 and 29 h, respectively. The great susceptibility of metronidazole benzoate to enzymatic hydrolysis is also apparent from its stability in the presence of plasma: in 80% human plasma (pH 7.4) at 37°C the half-life for the hydrolysis was found to be 3.6 min.

The possibility that the enzyme activity observed in the receptor phase originates from microbial contamination appears unlikely since the receptor phase contained a

Fig. 2.

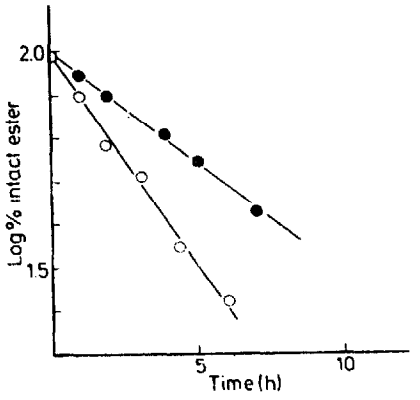

Fig. 2. First-order plot for the hydrolysis of metronidazole benzoate in receptor phase exposed to the human skin preparation in diffusion cells for 2.1 h (●) and 21.5 h (○) (37°C).

Fig. 3.

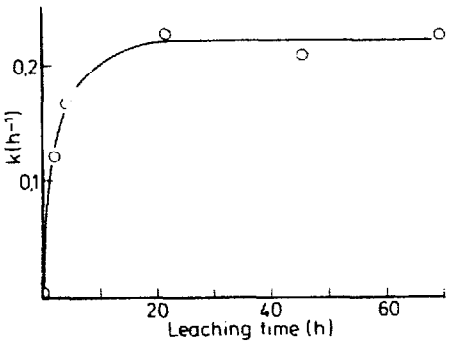

Fig. 3. Plot of the observed pseudo-first-order rate constant for the hydrolysis of metronidazole benzoate in receptor phase against leaching time.

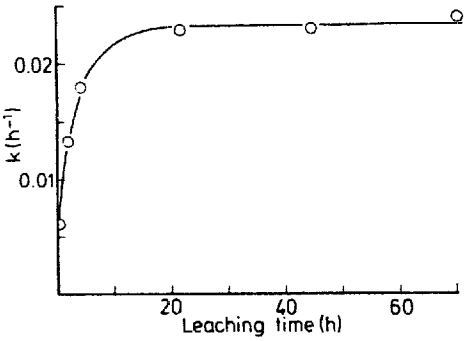

Fig. 4. Plot of the observed pseudo-first-order rate constant for the hydrolysis of 1-butyryloxymethyl-5-fluorouracil in receptor phase against leaching time.

preservative and the activity observed remained constant already after an exposure time of 20 h.

Permeation profiles

The permeation profiles obtained for metronidazole and its benzoate ester in terms of molar percentage in the receptor phase of the total amount applied to the excised human skin as a function of time are displayed in Fig. 5. The times required for the compounds to reach steady-state diffusion in the skin is about 20 h.

The permeability coefficient (k_p) representing the drug penetration rate, is given by (Scheuplein and Blank, 1971):

$$k_p = \frac{dq/dt}{A \cdot C_v} \qquad (1)$$

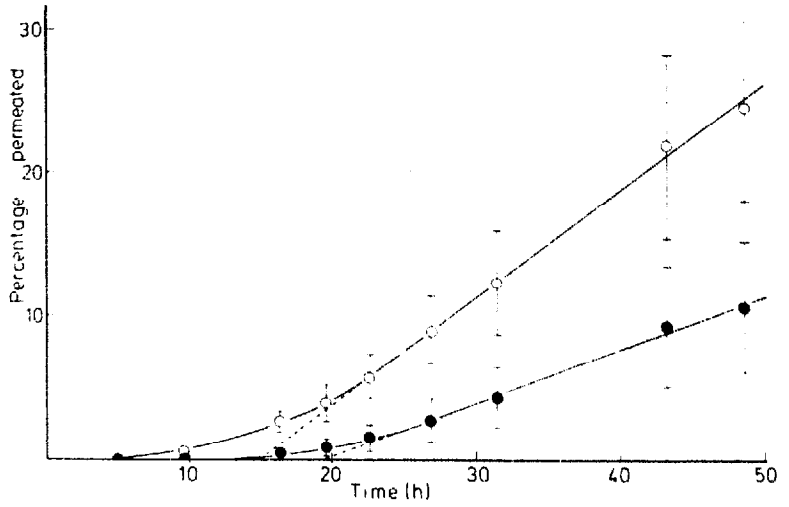

Fig. 5. Permeability of metronidazole (●) and metronidazole benzoate (○) through human skin as molar percentage appearing in the receptor phase relative to the total amount applied to the skin (175 μg in 10 mg of propylene glycol per cm^2). The data for metronidazole benzoate represent free metronidazole; no intact ester was detected in the receptor phase.

where dq/dt is the steady-state rate of penetration or appearance of the solute in the receptor phase, A is the area of the exposed skin and C_v is the concentration of the solute in the donor phase. From Eqn. 1 and the slopes of the linear portions of the plots in Fig. 5 the permeability coefficients were calculated to be 3.6×10^{-5} and 7.6×10^{-5} cm h^{-1} for metronidazole and the benzoate ester, respectively.

The enhanced skin permeability of the benzoate ester may most likely be ascribed to its more lipophilic character. According to Eqn. 2 (Scheuplein and Blank, 1971):

$$k_p = \frac{K_m D}{h} \tag{2}$$

where K_m is the skin (or stratum corneum)–vehicle partition coefficient of the solute and D is the solute diffusion constant in the skin barrier of thickness h, the permeability constant is proportional to the skin–vehicle partition coefficient. Assuming octanol to have the same solvent properties as stratum corneum (Roberts et al., 1975), the main diffusion barrier in the skin, the data in Table 1 show that the affinity to the skin in relation to the vehicle is greatest for metronidazole benzoate and that a proportionality, according to Eqn. 2, exists between k_p and the partition coefficient for the two compounds.

In the permeation experiments with metronidazole benzoate no unchanged ester was detected in the receptor phase (i.e. less than 0.05 $\mu g \cdot ml^{-1}$) during the study. This inability to detect the ester may be due to loss of the compound by hydrolysis in the receptor phase due to leached enzymes and/or conversion to metronidazole during the diffusion of the compound through the skin. Although the present data do not allow a strict analysis to be made of the relative magnitude of these two ways of ester hydrolysis it appears reasonable to state that at least some metabolism of the ester takes place in the skin during the permeation. If the benzoate ester permeated the skin unmetabolized with the determined flux of 1.33 $\mu g \cdot h^{-1} \cdot cm^{-2}$, and knowing that the hydrolysis in the receptor phase proceeds with a rate constant of 0.22 h^{-1} (Fig. 3), it can be calculated according to Loftsson (1982) that intact benzoate ester concentrations in the receptor phase would exceed 1 $\mu g \cdot ml^{-1}$. This

TABLE 1

SOLUBILITIES AND PARTITION COEFFICIENTS OF METRONIDAZOLE AND METRONIDAZOLE BENZOATE (at 22°C)

Compound	Solubility		Partition coefficients		k_p ($cm \cdot h^{-1}$)
	water (M)	propylene glycol (M)	octanol/water	octanol/propylene glycol [a]	
Metronidazole	0.050	0.11	0.85	0.38	3.6×10^{-5}
Metronidazole benzoate	0.00036	0.069	170	0.88	7.6×10^{-5}

[a] Calculated from the partition coefficient in octanol–water and the solubilities in water and propylene glycol.

concentration is 20 times higher than the detection limit in the HPLC assay. As discussed in a previous paper (Møllgaard et al., 1982) and supported by the present study, the appearance of 5-fluorouracil in the receptor phase after permeation of the 1-butyryloxymethyl derivative is predominantly due to cutaneous metabolism, the bulk phase metabolism playing only a minor role.

In conclusion, the results of this study show that hydrolytic enzymes are rapidly leached out from human skin preparations during the permeation study and this may hence lead to pronounced drug metabolism in the receptor phase. As exemplified with metronidazole benzoate such a kind of in vitro metabolism is important to take into account when assessing concurrent transport and metabolism of drugs in permeation studies using human skin. Whether the main hydrolysis of metronidazole benzoate is due to cutaneous or to bulk phase metabolism, the enhanced delivery characteristics along with the high susceptibility of the ester to hydrolysis by skin enzymes make such an ester type potentially useful in pro-drug design of drug molecules with hydroxyl groups with the purpose of improving the topical delivery characteristics.

References

Ando, H.Y., Ho, N.F.H. and Higuchi, W.I., In vitro estimates of topical bioavailability. J. Pharm. Sci., 66 (1977) 755–757.

Johansen, M., Bundgaard, H. and Falch, E., Spectrophotometric determination of the rates of hydrolysis of aldehyde-releasing pro-drugs in aqueous solution and plasma. Int. J. Pharm., 13 (1983) 89–98.

Loftsson, T., Experimental and theoretical model for studying simultaneous transport and metabolism of drugs in excised skin. Arch. Pharm. Chem., Sci. Edn., 10 (1982) 17–24.

Møllgaard, B., Hoelgaard, A. and Bundgaard, H., Pro-drugs as drug delivery systems. XXIII. Improved dermal delivery of 5-fluorouracil through human skin via N-acyloxymethyl pro-drug derivatives. Int. J. Pharm., 12 (1982) 153–162.

Roberts, M.S., Triggs, E.J. and Anderson, R.A., Permeability of solutes through biological membranes measured by a desorption technique. Nature (London), 257 (1975) 225–227.

Scheuplein, R.J. and Blank, I.H., Permeability of the skin. Physiol. Rev., 51 (1971) 702–747.

Yu, C.D., Fox, J.L., Ho, N.F.H. and Higuchi, W.I., Physical model evaluation of topical prodrug delivery — simultaneous transport and bioconversion of vidarabine-5'-valerate II: Parameter Determination. J. Pharm. Sci. 68 (1979) 1347–1357.

Yu, C.D., Fox, J.L., Higuchi, W.I. and Ho, N.F.H., Physical model evaluation of topical prodrug delivery — simultaneous transport and bioconversion of vidarabine-5'-valerate IV: Distribution of esterase and deaminase enzymes in hairless mouse skin. J. Pharm. Sci., 69 (1980) 772–775.