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International Journal of Pharmaceutics 236 (2002) 35–42

international journal of pharmaceutics

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The effect of ethanol on the simultaneous transport and metabolism of methyl *p*-hydroxybenzoate in excised skin of Yucatan micropig

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Received 9 August 2001; received in revised form 15 December 2001; accepted 25 December 2001

Abstract

The effects of ethanol on the simultaneous transport and metabolism of methyl *p*-hydroxybenzoate (HBM) were investigated in the skin of Yucatan micropig in vitro. It was found that transesterification occurred in the permeation studies involving ethanol. This was confirmed by monitoring the flux of ethyl *p*-hydroxybenzoate (HBE) into the receptor phase, as well as by monitoring the fluxes of HBM and *p*-hydroxybenzoic acid (HBA). The apparent flux of total HBM was decreased. The solubility of HBM increased with ethanol concentration, thus, the activity of HBM in ethanol solution became low because we used 10 mM HBM solution for permeation studies. The enhancement factor (E) was calculated to correct the activity. E increased with increasing the flux of ethanol, thus, ethanol may function as an enhancer of HBM transport. The hydrolysis of HBM to HBA was inhibited, whereas transesterification of HBM to HBE was induced at all concentrations of ethanol used (10–40%). The formation of HBE occurred much more readily than that of HBA at all concentrations of ethanol used.

Keywords: Skin metabolism; Skin permeation; Methyl *p*-hydroxybenzoate; Ethanol; Transesterification; Yucatan micropig skin

1. Introduction

Investigations into transdermal drug delivery systems have been actively researched with rapid development (Cleary, 1993). Great interest in using the dermal route for the administration of drugs, for both systemic and local effects, has strengthened the need for the investigation of vehicle effects on percutaneous absorption (Sloan and Koch, 1986; Mollgaard and Hoelgaard, 1983). Monitoring the transport and metabolism of a drug after topical application is necessary in order to establish a topical dosage form. For drugs that undergo biotransformation, skin metabolism may become a rate-limiting step during percutaneous absorption. Recently, enzymatic activity in the skin was investigated during a

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0378-5173/02/$ - see front matter
PII: S0378-5173(02)00005-4

transdermal therapy that relied on the bioconversion of a prodrug (Drustrup et al., 1991; Bonina et al., 1991; Liu et al., 1991).

Alcohols have been used as components of topical formulations probably since the first topical formulations were developed (e.g. lotions). Generally, there are two effects that alcohols and other formulation components have on the topical delivery of solute. Firstly, the presence of alcohol can affect the solubility of the solute in the formulation, and hence the ability of the solute to partition into the skin. Secondly, the movement of alcohol through the skin may affect the skin's permeability to the solute. This may result in irreversible damage to the skin by the alcohol and/or change the ability of the skin to solubilize the solute (Sloan et al., 1998). Ethanol has been reported to enhance the permeation of several drugs (Ghanem et al., 1987; Kai et al., 1990; Sloan et al., 1998). Liu et al. (1991) suggested that ethanol functions as an inhibitor of the enzymatic conversion of used drug in the viable epidermis, and that ethanol is able to enhance the transport of permeants across the lipoidal pathway of the stratum corneum.

In the present study, methyl *p*-hydroxybenzoate (HBM) was selected as a model drug in order to investigate the effects of ethanol on the transport and metabolism of drug in skin. HBM is widely used in the formulations for external use as preservatives and stable in the solution. Moreover, metabolism of *p*-hydroxy benzoic acid esters was observed in human skin (Lobemeier et al., 1996). Yucatan micropig (YMP) skin was used as an animal model for our in vitro percutaneous studies as it possesses similarities to human skin in the views of immunohistochemical (Lavker et al., 1991) and permeation of drugs (Fujii et al., 1997; Rohatagi et al., 1997). We found that transesterification of HBM in YMP skin occurred in all permeation experiments using ethanol. This was confirmed by monitoring the flux of ethyl *p*-hydroxybenzoate (HBE) into the receptor phase as well as investigating the fluxes of HBM and *p*-hydroxybenzoic acid (HBA). The objectives of this present study were threefold. Firstly, to examine the enhancing effect of ethanol on the transport of HBM through excised YMP skin. Secondly, to investigate the presence of transesterified molecules in both excised and homogenized forms of YMP skin. And thirdly, to detail any inhibition in conversion of HBM to HBA and HBM to HBE.

2. Materials and methods

2.1. Materials

HBA was obtained from Nakarai Chemical Ltd. (Kyoto, Japan). HBM, HBE and ethanol were obtained from Wako Pure Chemical Industries (Osaka, Japan). Skin samples (obtained from 5 month old, female YMPs) were purchased from Charles River Japan, Inc. (Yokohama, Japan) in the frozen state at -80 °C. Other chemicals and solvents were of reagent grade and were used without further purification.

2.2. In vitro skin permeation study

A piece of skin was thawed at room temperature for approximately 30 min, followed by removal of the adhering fat layer using scissors and a grater. Stripped skin was obtained by removing the stratum corneum using adhesive cellophane tape (Washitake et al., 1973). The skin was cut to an appropriate size and mounted on modified Franz-type diffusion cells (1.1 cm^2). The receptor compartment was filled with pH 7.1 isotonic phosphate buffered saline (PBS) (≈ 16 ml) containing 0.01% kanamycin and kept at 37 °C with stirring at 600 rpm. An aliquot (3 ml) of the drug solution was applied to the donor compartment. A glass ball occluded the upper portion of the donor phase. At appropriate time intervals, 0.2 ml of the receptor phase was withdrawn, and the same volume of fresh PBS was added to the cell to keep the volume constant. Each experiment was carried out for 30 h. It was confirmed that the enzyme activity was kept about 90% after 30 h. After which time, the skin was removed from the diffusion cell apparatus and wiped with purified water. The epidermis, consisting of the stratum corneum and viable epidermis, was separated from the dermis by a heat separation technique

(Kligman and Christophers, 1963). The separated tissues were then minced with scissors and homogenized using a Hiscotoron NS-50 (Nichion, Chiba, Japan) homogenizer following the addition of 75% methanol. After centrifugation, the supernatant of each was injected into an HPLC system.

2.3. Metabolism study in skin homogenate

The effect of ethanol on the hydrolysis of HBM to HBA and the formation of HBE from HBM was investigated in skin homogenates. One gram of full-thickness skin was homogenized in 10 ml of PBS at 4 °C and the homogenate was then centrifuged at 3000 rpm. The supernatant was filtered through glass wool to remove any particulate matter and then stored in several aliquots at − 20 °C until needed. The protein content of the supernatants was measured by Bio-Rad Protein Assay (Bio-Rad, USA) based the method of Bradford (1976) using bovine γ-globulin as the standard. Metabolic activity was determined by monitoring the formation of HBA and HBE from HBM in skin homogenates. To 5 ml of the skin supernatant solution previously equilibrated at 37 °C was added 0.5 ml of HBM solution. This was incubated in a shaker bath at 37 °C. At predetermined time points a 0.2 ml aliquot was withdrawn, and the reaction immediately stopped by the addition of 0.8 ml ice-cold methanol followed by thorough mixing. The solution was then centrifuged at 3000 rpm for 15 min and the supernatant was analyzed by HPLC. The formation rates of HBA and HBE, calculated as the number of nanomoles of product formed per milligram of protein, was calculated from the initial slopes of formation plots of HBA and HBE versus time. The control experiments, in which skin homogenates were replaced with pure buffer solutions, showed no measurable amounts of HBA or HBE, indicating that no significant production of these products occurred during the time span of the analyses.

2.4. Solubility test

Excess amounts of HBM were introduced into 2 ml of PBS containing various concentrations of ethanol and sealed with Parafilm. The tubes were shaken for 48 h at 37 °C in a water bath and then centrifuged at 3000 rpm for 15 min. The drug concentration in the clear supernatant solution was determined by HPLC.

2.5. Analytical method

HBA, HBM and HBE were determined by HPLC. The apparatus consisted of a pump (LC-10AD, Shimadzu, Kyoto, Japan), an ultraviolet detector (SPD-6A, Shimadzu), a 4.6 mm × 150 mm stainless steel column packed with TSK-Gel ODS-120T (Tosoh, Tokyo, Japan) and an integrator (C-R6A, Shimadzu). The mobile phase consisted of methanol: 0.2% phosphoric acid (4:6, v/v) and had a flow rate of 1.0 ml/min. HBA, HBM and HBE were detected by monitoring the absorbance of the eluent at 264 nm.

Ethanol was determined using a GC system fitted with a FID detector (Shimadzu GC-14B). A polyimide coated fused silica capillary column (HR 20M-PM25 0.25 Shinwakako, Japan) was used. Nitrogen was used as carrier gas (100 kPa). The column, injection port and detector temperatures were controlled at 130, 200 and 200 °C respectively.

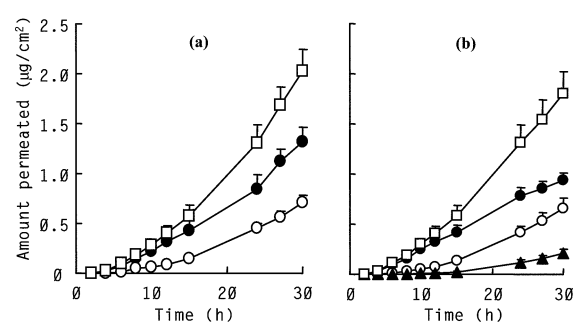

Fig. 1. Cumulative amounts of HBA, HBE and HBM permeated through full-thickness skin of YMP after application of 10 mM HBM without (a) and with 10% ethanol (b). HBM (○), HBA (●), HBE (▲), HBM_{total} (□). Each point represents the mean ± S.E. of at least six experiments.

Table 1
The flux of HBA, HBE, HBM_{total} and ethanol through excised YMP skin after application of an aqueous solution of HBM (10 mM) containing various concentrations of ethanol in the donor compartment

Skin condition	Ethanol (%)[a]	Flux[b]			
		HBA (nmol/cm^2/h)	HBE (nmol/cm^2/h)	HBM_{total}[c] (nmol/cm^2/h)	Ethanol (μmol/cm^2/h)
Full-thickness skin	0	44.2 ± 6.3	–	70.8 ± 8.8	–
	10	38.4 ± 3.7	7.6 ± 1.4	70.9 ± 9.2	3.4 ± 0.0
	20	24.6 ± 5.0	16.2 ± 2.8	61.8 ± 10.0	10.3 ± 1.7
	30	14.8 ± 2.3	4.4 ± 0.9	23.1 ± 1.8	13.7 ± 1.7
	40	9.4 ± 1.0	3.4 ± 0.0	15.1 ± 0.8	15.4 ± 1.7
Stripped skin	0	67.4 ± 0.4	–	202.9 ± 4.5	–
	10	24.6 ± 8.0	48.0 ± 8.2	301.5 ± 17.0	28.7 ± 2.2
	20	22.9 ± 2.8	52.9 ± 5.7	178.7 ± 4.4	46.3 ± 13.7
	30	13.4 ± 0.9	38.9 ± 5.8	109.8 ± 9.9	61.7 ± 12.0
	40	18.8 ± 1.9	32.4 ± 4.7	80.3 ± 11.0	80.7 ± 3.3

[a] The concentration of ethanol in donor compartment.
[b] Each value represents the mean ± S.E. of at least six experiments.
[c] HBM_{total} represents the sum total of HBA, HBE and HBM permeated.

3. Results and discussion

Fig. 1 details the in vitro permeation and metabolism profile of HBM in the presence of excised YMP skin after application of an aqueous solution of HBM at a concentration of 10 mM in the donor compartment of a diffusion cell set. In the absence of ethanol, the cumulative amounts of HBM and HBA increased linearly with time, although a lag time was found (Fig. 1(a)). In the presence of 10% ethanol, a new flux of HBE was observed and the flux of HBA decreased (Fig. 1(b)), suggesting that a transesterified form of HBM resulted due to the presence of ethanol.

This experiment was repeated using different concentrations of ethanol in the donor compartment. Table 1 represents the steady-state flux of HBA, HBE, HBM_{total} (the sum of HBM, HBA and HBE) and ethanol through full-thickness and stripped skin samples of YMP. These results clearly indicate that the stratum corneum layer greatly influences the solute transport behavior of skin. It is well known that ethanol has an enhancing effect on the transport of permeants, nevertheless the total flux of HBM permeated decreased with increasing concentration of ethanol. The solubility of HBM increased with increasing ethanol concentration (Table 2). We used 10 mM HBM solution in permeation studies, thus the activity of HBM in the donor phase decreased with increasing ethanol concentration. The low activity read low partition to the skin, so the apparent flux of HBM decreased. The enhancement factor, E, was demonstrated by Ghanem et al. (1987) to compare the fluxes from different activity solution. It was calculated as follows:

$$E = (P_{sc} \text{ with ethanol})/(P_{sc} \text{ without ethanol}) \times (S_{ethanol})/(S_{water})$$

$$1/P_{full} = 1/P_{sc} + 1/P_{stripped}$$

where P_{sc}, P_{full} and $P_{stripped}$ represent the permeation coefficients through the stratum corneum,

Table 2
The solubility of HBM in phosphate buffered saline (pH 7.1) containing various concentrations of ethanol at 37 °C

Ethanol (%)	Solubility of HBM (mM)
0	11.3 ± 1.1
10	26.9 ± 0.8
20	58.5 ± 1.7
30	223 ± 3
40	590 ± 29

Each value represents the mean ± S.D. of three experiments.

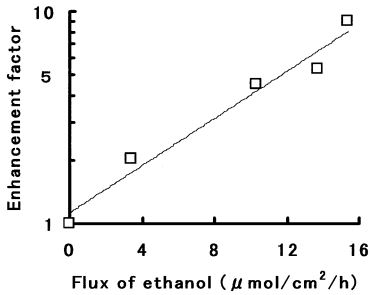

Fig. 2. The enhancement factor, E, on the stratum corneum layer of the excised YMP skin as a function of ethanol flux.

full-thickness skin and stripped skin, respectively. S_{water} and $S_{ethanol}$ represent the solubility of HBM in water and ethanol, respectively.

As shown in Fig. 2, there is a semi-log-linear relationship between E and the ethanol flux. This coincides with the results of Ghanem et al. (1987). In the presence of 40% ethanol the enhancement factor was approximately 9. This was the same as in the cases when indomethacin and antipyrine were used (data not shown).

The flux of HBA also decreased with increasing ethanol concentration. The maximum flux of HBE was observed when the ethanol concentration was 20%. The flux of the sum of HBA and HBE (HBA + HBE) decreased with increasing ethanol concentration, just like the flux of HBM_{total}. The ratios of (HBA + HBE)/HBM_{total} were 60–80% in the case of full-thickness skin, whereas 20–60% in the case of stripped skin. Thus, the relationship between the flux of HBM_{total} and the flux of HBA + HBE was examined (Fig. 3(a)). The flux of HBA + HBE increased with increasing the flux of HBM_{total} when it was low value, however, the flux of HBA + HBE was not increased when the flux of HBM_{total} became high. Fig. 3(b) is reciprocal plot like Lineweaver–Burk plot. It shows good correlation. These suggest that limiting step of permeation of HBA + HBE is permeation of HBM_{total} in the case of full-thickness skin, however, metabolism is limiting step when the permeation of HBM_{total} is extremely high like through stripped skin and also that ethanol shows no effect on the enzyme activity. The flux of HBA through full-thickness skin and that through stripped skin were not so different but the flux of HBE through stripped skin was obviously higher than that through full-thickness skin. It may be because of high permeation of ethanol through stripped skin.

In an attempt to shed light on the metabolism of HBM in the presence or absence of ethanol, the hydrolysis of HBM in skin homogenates was examined. There was no formation of HBA and HBE from HBM solution without skin homogenates. When HBA solution was added to skin homogenates containing ethanol, no HBE was detected. This suggests that the formation of HBE occurred via a pathway from HBM to HBA. Fig. 4 shows the Hanes–Woolf plots of metabolite in the presence and absence of ethanol. In both cases, the plots suggest the presence of an enzymatic reaction. The Michaelis–Menten constant (K_m) and the maximum reaction rate (V_{max})

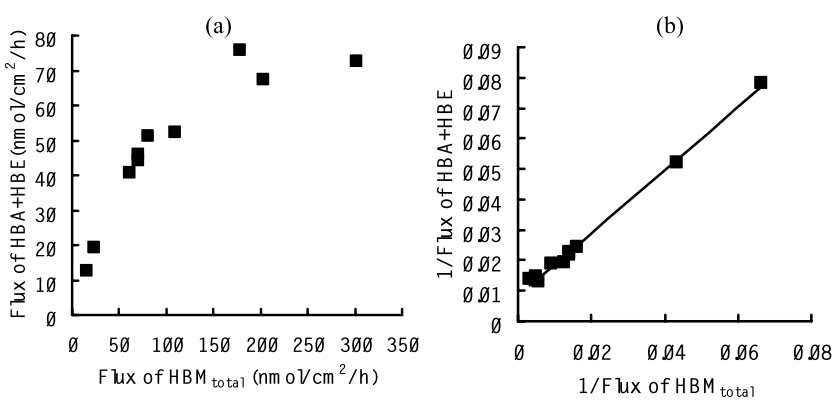

Fig. 3. Relationship between flux of HBM_{total} and flux of HBA + HBE (a) and its reciprocal plot (b).

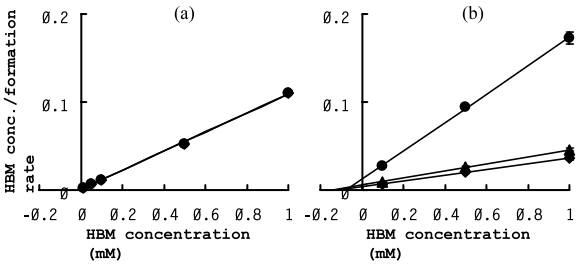

Fig. 4. Hanes–Woolf plot depicting the hydrolysis of HBM to HBA and the transesterification of HBM to HBE, in skin homogenates without (a) and with 1% ethanol (b). HBA (●), HBE (▲), HBA + HBE (◆). Each point shows the mean ± S.E. of at least three experiments.

for the metabolism of HBM to HBA in skin were estimated to be 23 μM and 9.4 nmol/h/mg protein, respectively (Fig. 4(a)). In the presence of 1% ethanol, K_m and V_{max} were calculated to be 76 μM and 6.2 nmol/h/mg protein, respectively (Fig. 4(b)). These parameters can be used as an index for evaluating the metabolic activity in skin. Our results indicate that ethanol inhibits the metabolism of HBM to HBA in skin homogenates. In the case of HBE formation from HBM in skin homogenates containing 1% of ethanol, K_m and V_{max} were 130 μM and 24.6 nmol/h/mg protein, respectively. These results indicate that the formation of HBE from HBM occurs more readily in skin homogenates containing 1% ethanol.

Fig. 5 shows the effect of ethanol on the hydrolysis of HBM to HBA and the transesterification of HBM to HBE. The formation of HBA decreased with increasing ethanol concentration. The sum of HBA and HBE detected increased with ethanol concentrations in the range 0.5–2%, but decreased remarkably when the ethanol concentration was over 10%. This suggests that enzymatic activity is inhibited by high concentrations of ethanol, but it is not inhibited by ethanol in concentrations up to 5%. Interestingly, the formation rate of HBE was higher than that of HBA at all concentrations of ethanol used.

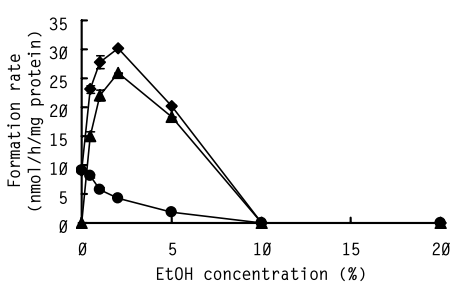

Fig. 5. The effects of ethanol on the hydrolysis of HBM to HBA and the transesterification of HBM to HBE in skin homogenates. HBA (●), HBE (▲), HBA + HBE (◆). HBM concentrations were fixed to 1 mM. Each point represents the mean ± S.E. of at least three experiments.

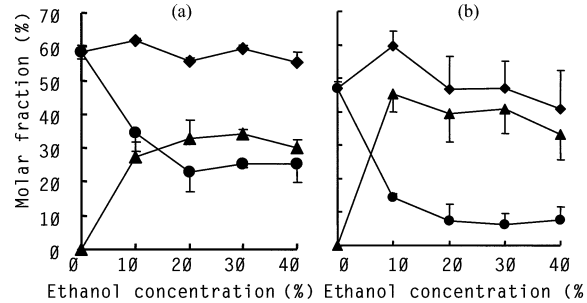

Fig. 6. The formation ratio of HBE and HBA from HBM in the skin permeation studies containing varying amounts of ethanol calculated from total amount (b). Calculated from amount in skin. HBA (●), HBE (▲), HBA + HBE (◆). Each point represents the mean ± S.E. of at least three experiments.

The presence of HBE as the major formation product in skin homogenates was not consistently observed in our in vitro permeation studies through full-thickness skin (Table 1). There are two possibilities; one, low ethanol concentration in the skin and another, the difference of lipophilicity between HBA and HBE. Fig. 6(a) shows the molar fraction of HBA and/or HBE at 30 h. The molar fraction was calculated as (total amount of HBA and/or HBE present in the donor phase, epidermis, dermis and receptor phase)/(total amount of HBM, HBA and HBE present in the epidermis, dermis and receptor phase, and HBA and HBE in the donor phase). The sum molar fraction of HBA and HBE was approximately 60%, even at ethanol concentrations up to 40%. This indicates that 40% ethanol did not inhibit the enzyme activity in the skin permeation studies. The ratio of HBE to HBA was found to be approximately 1, and appeared to be independent of ethanol concentration, suggesting suffi-

cient ethanol concentration in the skin. This phenomenon could result due to HBE having a higher lipophilicity than HBA, as reflected by the lower partitioning of HBE to the receptor phase. In accordance with this supposition, the concentration of HBE in the skin was higher than that of HBA (Fig. 6(b)).

4. Conclusion

Ethanol functioned as an enhancer of HBM transport, even though the apparent flux of total HBM decreased in the presence of ethanol. Ethanol also inhibited the hydrolysis of HBM to HBA in the skin of YMP. The transesterification of HBM to HBE was detected in our permeation studies of HBM, as well as in YMP skin homogenates containing ethanol. However, HBE could not be detected in these same experiments of HBA. These results suggest that drug molecules containing an ester group may be converted to other ester forms when alcohol is used as a vehicle. Consequently, unexpected outcomes may result. In Fig. 7 we propose a possible pathway for the hydrolysis and transesterification of HBM. In the presence of ethanol, both HBA and HBE are formed enzymatically. HBE is a major product, however, lipophilic character of HBE prevents its partition to the receptor phase. Further studies are necessary in order to elucidate the nature of the ester modification of a drug in the presence of alcohol.

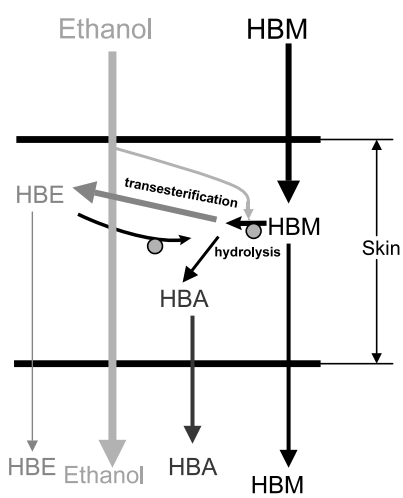

Fig. 7. Schematic diagram depicting the possible formation of HBA and HBE from HBM in YMP skin studies containing ethanol

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