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Lipid Extracting Effect of Ethanol on Keratinized Oral Mucosa

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INTRODUCTION

Ethanol has been widely used as a percutaneous penetration enhancer and its action on the cutaneous barrier has been well studied. Ethanol may have a "fluidisation" effect on the intercellular lipids of the stratum corneum, the insertion of ethanol between endogenous lipid provokes an increase in structural disorder. This effect has been reported to occur at low ethanol concentrations (25% v/v) (1) and has been compared to the effect of increasing skin temperature (2).

An increasing extraction of epidermal lipids with increasing ethanol concentrations, has also been reported as the major mechanism by which alkanols promote transdermal penetration (3). It has been observed that very high alkanol concentrations (> 75%), result in significant lipid extraction and with 100% ethanol treatment, full thickness skin appears to be devoid of its barrier and even behaves like stripped skin (1,4). Both contact time and ethanol concentration influence whether or not the effects of ethanol treatment are reversible (1,3–5). As in the skin, the action of ethanol on oral mucosa depends upon the amount of alcohol in contact with the membrane and the contact time (6). As the ethanol concentration is increased, there is a greater lipid extraction, accompanied by a reorganization of the intercellular lipids. Therefore, one can expect that treatment with high ethanol levels for long periods, will produce a greater perturbation of the membrane.

Based on the evidence described above, in this study we have addressed the following objectives: (a) To determine the action of ethanol as a lipid extracting solvent, on keratinized oral mucosa. (b) To separate, identify and quantify the lipids present in the hydroalcoholic treating solutions. (c) To determine the relative importance of ethanol concentration and contact time on lipid extraction.

MATERIALS AND METHODS

Sample Preparation

(a) Palatal tissue from male and female pigs was obtained from the slaughterhouse. After detachment from the bone, the submucosal tissue of the palate was carefully removed. The tissue was then frozen under liquid nitrogen and kept at −20°C before use (no more than 2 weeks). In order to see the effect of ethanol on the tissue, pieces of the sample were defrosted in isotonic saline solution and clamped into position between the donor and the receptor compartment of a static vertical diffusion cell, based on the Franz design (exposed area = 0.78 cm^2). The receptor medium, consisting of 2.2 ml of isotonic saline solution, was maintained at 37°C by a circulating water pump, and constantly stirred with a teflon-coated magnetic bar. The stratum corneum side of the tissue was hydrated for 30 min, with the receptor medium and subsequently dried with a pre-cleaned (delipidized) cotton swab. Then, 0.5 ml of the hydroalcoholic solution was applied. The donor solution was withdrawn from the diffusion cell and was subsequently analysed by HPTLC for lipid content. Different hydroalcoholic solutions (0, 25, 50, 75 and 100% v/v) and contact times (0.17, 1, 3, 6, 24 h) were randomly assayed.

(b) Additionally, a lipid extraction was carried out by exposing, consecutively, a known weight of dry stratum corneum to chloroform/methanol 2:1; 1:1 and 1:2 v/v. Samples were also analysed by HPTLC.

Chromatographic Separation

The donor solutions were dried under vacuum and the residuals reconstituted with 0.2 ml of chloroform:methanol (2:1). Samples were filtered through 0.45 μm polypropylene micro-centrifuge tube filters (Whatman, England) and 15 μl was spotted on high performance silica gel 60 thin-layer chromatography plates, without concentration zone (Merck, Darmstadt, Germany), using a Linomat IV (CAMAG, Muttenz, Switzerland). Separations were carried out in developing chambers (CAMAG), saturated with the appropriate solvent system, at room temperature. Three consecutive separation steps were carried out, using the following solvent systems, according to Melnik et al. (7): (a) methanol:chloroform:water (20:95:1); (b) n-hexane:diethyl ether:glacial acetic acid (80:20:10); (c) petroleum ether. The solvent front was allowed to migrate to 6.0 cm, 8.5 cm and to full length above the origin, respectively for

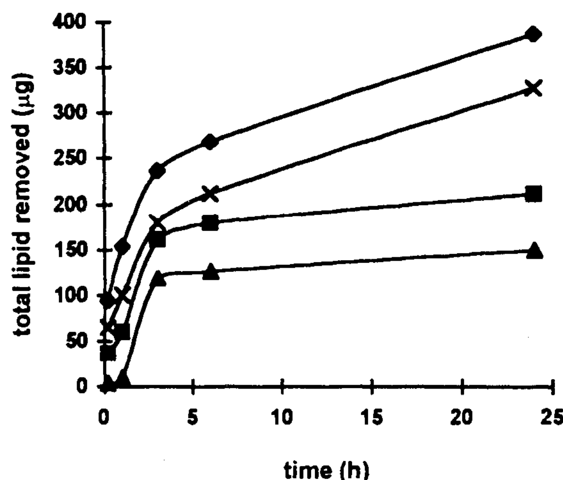

Fig. 1. Effect of contact time on the lipid leaching from palatal mucosa. ♦ 100 %; x 75 %; ■ 50 %; ▲ 25 % (n = 6).

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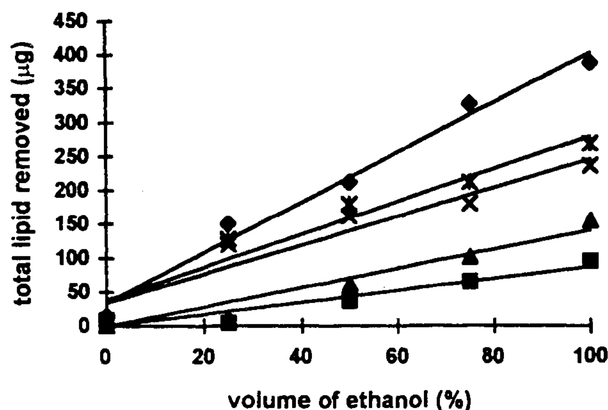

Fig. 2. Effect of ethanol concentration on the lipid leaching from palatal mucosa. ◆ 24 h; * 6 h; x 3 h; ▲ 1 h; ■ 0.17 h (n = 6).

the three solvent systems. After drying the plates, they were sprayed with cupric sulphate pentahydrate (10% w/v) in phosphoric acid (8% w/v) and charred on a hot plate (CAMAG), by progressively heating to 140°C for 15 min. Finally, the plates were scanned with a CAMAG TLC Scanner II at 420 nm, in the Refl-Abs mode, and the signals were integrated with a CAMAG SP4290 integrator. Lipid standards were used on each plate for mass calibration purposes: squalene, cholesterol-3-sulphate, cerebroside type II, ceramide type III, ceramide type IV, cholesterol, triolein, oleic acid and cholesteryl oleate, provided by Sigma (St. Louis MO, USA). Soya-bean lecithin (Epikuron 170) was purchased from Lucas Meyer (Hamburg, Germany).

RESULTS AND DISCUSSION

Analysis of the hydroalcoholic treatment liquid showed that considerable lipid extraction from porcine palatal mucosa had occurred, the full spectrum of stratum corneum lipids being shown to be present. Numerous studies with epidermal stratum corneum treated with ethanol, have reported the presence of lipids in the treatment liquid, as well as an appreciable reduction of the stratum corneum lipid-content (3,4).

As expected, we found that treatment for long periods and with high ethanol levels, produced a greater extraction. As shown in Fig. 1, the amount of lipid removed, increased sharply as a function of time. For longer exposure times, extraction increased moderately with 75 and 100% ethanol but did not show an appreciable increase with lower ethanol concentrations (25 and 50%). It is also interesting to note that the amount of lipid in the treatment solutions increased linearly with ethanol content (Fig. 2). Prolonged contact with oral mucosa, may result in the extraction of membrane lipids with a reorganization of the lipid domains, most probably compromising membrane barrier function.

We considered it important to show the amount of each individual lipid group extracted as a function of ethanol level and contact time, because this may give an indication about which lipid classes were most affected by this treatment. As shown in Table 1, the order of extraction according to the amount of lipid found in the treatment liquid, for 100% ethanol after 24 h contact was: cholesterol (CH) > sphingolipids (SF) > fatty acids (FA) > triglycerides (TG) > phospholipids (PP) > cholesterol sulphate (CS) > cholesterol esters (CE). The results in Fig. 3, show that some lipid classes were more affected by ethanol, requiring a lower ethanol content to be extracted, while for others, a higher concentration or contact time was necessary. As shown, the extracted amount of CH, SF, FA and TG was considerable for both, moderate exposure times (3 h) and low ethanol levels (25%). For PP and CS, a high volume of ethanol (> 75%) was necessary to observe a significant extraction; low ethanol levels (< 50%) did not effect pronounced extraction of these lipids, even for long contact times (24 h). Long exposure time (24 h) was necessary to extract CE with 25% ethanol, however, higher ethanol levels (≥ 50%) led to a significant extraction, even at short times.

Different explanations can be proposed to account for these observations. At first, as shown in Table 1, the lipids present in the 100% ethanolic solution in major quantity (CH, SF and TG), are also some of the principal components of the membrane (10), with the exception of FA, which were found in an unusually high proportion with respect to the amount in the chloroform/methanol mixture. However, it should be pointed out that the extraction was not carried out under the same conditions, and

Table 1. Lipid Content (weight %) After Extraction with 100% Ethanol for 24 h and with Chloroform/Methanol, and the Solubility Parameter Values (δ)

Lipids	Chloroform/methanol	Ethanol	δ ($cal^{1/2}cm^{-3/2}$)[a]
Cholesterol	59.82 ± 11.61	33.51 ± 8.22	5.89 (9.75)[g]
Sphingolipids	6.87 ± 3.88	22.20 ± 7.43	12.72[b]
Triglycerides	9.22 ± 1.24	12.47 ± 4.83	8.38[c]
Phospholipids	18.97 ± 2.8269	7.39 ± 3.85	9.42[d]
Cholesterol sulfate	1.31 ± 0.19	2.50 ± 0.83	not calculated
Fatty acids	2.44 ± 0.07	20.01 ± 7.82	8.51; 8.20[e] (7.97; 8.05)[g]
Cholesterol esters	1.36 ± 0.67	1.90 ± 0.47	6.99[f]

[a] Calculated according to Small's molar attraction constants (8).
[b] Value for ceramide II.
[c] Value for triolein.
[d] Value for lecithin, considering it was composed of palmitic chains.
[e] Value for oleic and palmytic acid, respectively.
[f] Value for cholesteryl oleate.
[g] In brackets, reported parameter (9).

that probably the presence of the stratum corneum-underlying tissue, in the case of ethanol, contributed to this increased extraction.

The possibility of a specific interaction between ethanol and the different lipids, should not be ruled out, since this has been demonstrated for some phospholipids (11). It has been reported that the level of ethanol may also affect the lipids in different ways, in this respect, Coutel-Egros et al. (12), proposed that at low concentrations (< 30%), ethanol interacts with the polar domains of lipids, whereas at a concentration of 30% there is an interaction with the nonpolar lipid domains. A biphasic effect was observed in liposomal models treated with ethanol (11). Depending on its concentration, ethanol can interact in two different ways with phosphatidylcholine bilayers: at low concentration, there is a linear decrease of the T_M, explained by more ethanol partitioning in the liquid-crystalline than in the gel phase. At a particular threshold ethanol concentration (~40–60 mg/ml for phosphatidylcholine), there is an abrupt change in T_M, which marks the induction of an irreversible transition from a normal gel phase, to an interdigitated state, in which ethanol partitions preferentially into the gel phase. The induction of this state by ethanol, may change the properties of the membrane at interfaces or boundaries between the different phases, altering in this way the arrangement and interactions between lipid molecules.

It has already been reported that vehicles with a solubility parameter (δ) similar to the tissue cause the most damage, because they are able to solubilize the components of the membrane responsible for barrier function (13). The complex composition of the tissue makes it difficult to determine its solubility parameter. A solubility parameter of 9.7–10.0 H ($cal^{1/2}/cm^{3/2}$) for intact stratum corneum, was calculated by Liron and Cohen (14). We have made a rough estimation of δ for some standard lipids and, as shown in Table 1, we found values between ~6 and 12 H. However, it is difficult to define a clear relationship between the calculated solubility parameters for each individual group of lipids and the corresponding amount extracted. Firstly, because the values calculated for these few standard lipids, do not represent in reality the great variety of lipids found in the tissue; and because of the uncertainty in the calculations to predict δ, due to the differences in the values of molar attraction constants given by the different authors (8). We can speculate, for example, that sphingolipids were extracted in a great quantity because of their high solubility parameter, very close to the ethanol solubility parameter. A δ ~12 H, was calculated for ceramide II, the most abundant sphingolipid in palatal stratum corneum (10). The solubility parameters for the other lipids are relatively close to one another, and therefore, we cannot explain why some of the lipids appear to be more easily extracted than others simply by their δ values.

In general, the addition to water (δ = 23.4 H) of an organic cosolvent, such as ethanol (δ = 12.8 H), will decrease the solubility parameter of the solvent mixture, with a resulting increase in the quantity of lipid it is capable of dissolving. Then, solubility might increase progressively with increasing ethanol concentration (8). This could explain the linear relationship between the amount of lipids removed and the ethanol content (Fig. 2). On the other hand, a cosolvency ethanol:lipids, due to their similar δ values, ensures not only the solubilization of lipids in ethanol, but also a relatively high solubility of ethanol in the lipid domains.

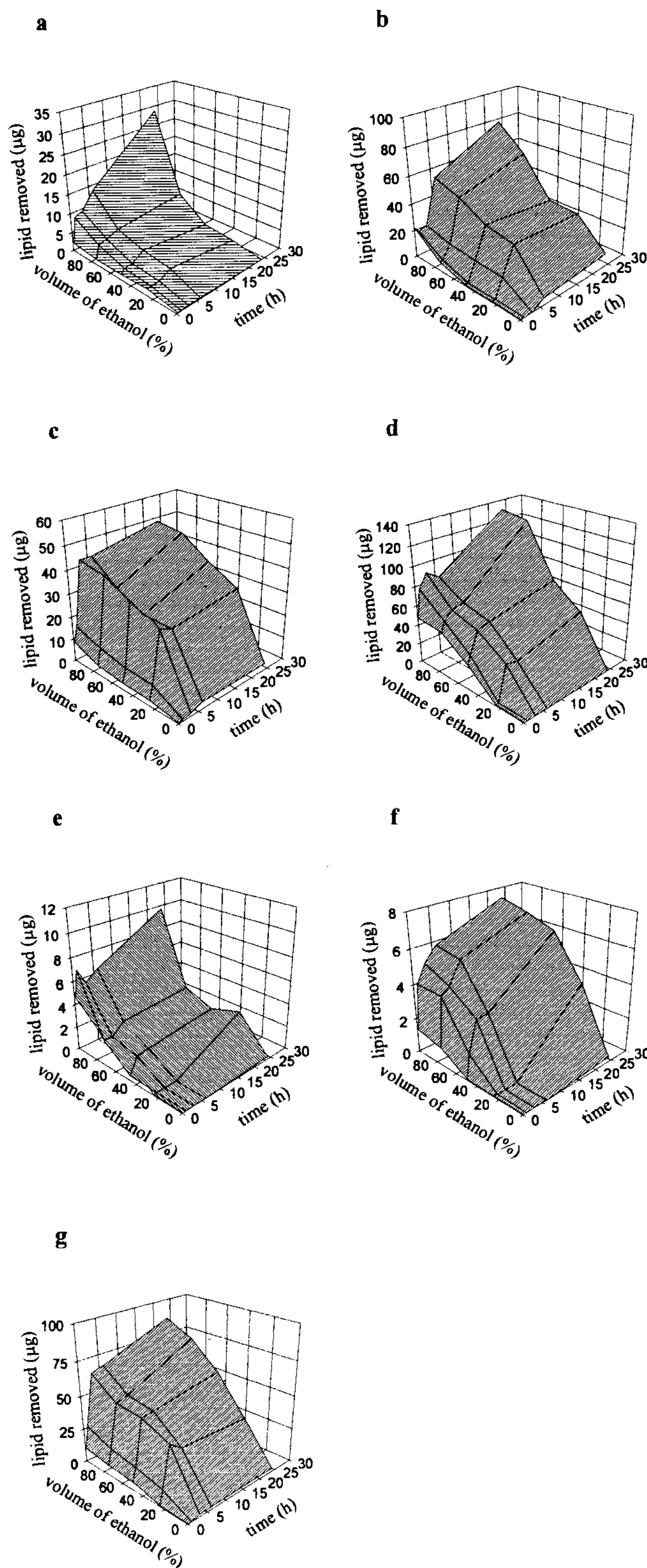

Fig. 3. Extracted amount of lipids from palatal mucosa as a function of contact time and ethanol concentration. (**a**) phospholipids; (**b**) fatty acids; (**c**) triglycerides; (**d**) cholesterol; (**e**) cholesterol sulphate; (**f**) cholesterol esters; (**g**) total sphingolipids.

However, the action of ethanol on stratum corneum lipids is quite complex and as mentioned above, different effects which appear to be concentration and time dependent, have been proposed by several authors (11–12). The fact that some lipid classes were apparently more easily removed than others, may be a consequence of several factors, including intermolecular organization. It has been proposed that the presence of ethanol can provoke a reduction of lipid polar head-group interactions, it is able to induce lipid phase segregation and it can cause the redistribution of some lipids within the membrane (*flip-flop*) (2).

In addition, lipids are generally considered as being bilayer-structured, but in fact, they can adopt other conformations. Phospholipids like sphingomyelin have one or more metastable conformations at physiological temperatures, depending on the degree of hydration (15). Thus, a solvent, like ethanol, that is able to change hydration, may induce conformational changes, thereby facilitating extraction. Moreover, the ability of polar lipids to form micelles, may also play a role in the solubilization process of other lipid entities.

These findings may be different for nonkeratinized regions, where lipid composition differs from that found in keratinized epithelia. Results cannot be readily extrapolated to *in vivo* situations, because of factors such as salivary flux and salivary mucin coating can alter local concentration.

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