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Safety Aspects of Non-ionic Surfactant Vesicles: A Toxicity Study Related to the Physicochemical Characteristics of Non-ionic Surfactants

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Abstract—Two different toxicity models were used to assess the relationship between the physicochemical properties of non-ionic surfactant vesicles (NSVs), and the safety of these vesicles for topical drug administration. The vesicles used in this study consisted of polyoxyethylene alkyl ethers (C_nEO_m) in which the number of C atoms (n) varied between 12 and 18 and the number of oxyethylene units (m) between 3 and 7. The physicochemical properties of the vesicles are described in terms of hydrophilic-lipophilic balance (HLB) values, and critical micelle concentrations (CMC), and the rigidity of the bilayers as determined by the gel-liquid transition temperatures and the cholesterol content of the bilayers. The first toxicity model, comprising the measurement of the ciliary beat frequency, is a tool to assess the safety of intranasally applied formulations. Studies using this ciliotoxicity model revealed that by increasing the length of the alkyl chain of the surfactant, a decrease in toxicity was observed. The opposite correlation was found if the length of the polyoxyethylene headgroup was increased. Furthermore, it was observed that gel-state vesicles produce less of an effect on the ciliary beat frequency than liquid state vesicles. The second toxicity model, comprising the determination of cell proliferation of human keratinocytes, is a method to assess skin irritancy. In contrast to the ciliotoxicity model the length of the polyoxyethylene headgroup and of the alkyl chains did not seem to have an effect on the safety of the vesicles. However, the bond by which the headgroup is linked to the alkyl chain, showed a very strong effect on the toxicity of the surfactant: oleyl-EO_5 ester vesicles were found to have an effect on the cell proliferation, which was one-sixteenth that of the oleyl-EO_5 ether vesicles. The cholesterol content did not appear to have an effect on the proliferation of the keratinocytes. Neither the HLB nor the CMC values appeared to have an effect on the safety of the NSV formulations as observed in both toxicity models.

Phospholipid vesicles, or liposomes, have been extensively studied as possible carriers in intravenous drug targeting, in order to alter the distribution and toxicity of encapsulated bio-active compounds. Handjani-Vila et al (1979) introduced a new type of vesicle prepared from non-ionic surfactants. Various types of surfactants have been used for the preparation of non-ionic surfactant vesicles (NSVs), such as polyglycerol alkyl ethers (Handjani-Villa et al 1979; Baillie et al 1985), glucosyl dialkyl ethers (Kiwada et al 1985a), crown ethers (Echegoyen et al 1988) and polyoxyethylene alkyl ethers (Hofland et al 1988, 1989, 1990, 1991). Only a few studies concerning NSVs, involving animals, have been reported (Ozer et al 1991). In mice the distribution of drugs incorporated in NSVs has proved to be similar to that obtained after administration of drugs encapsulated in liposomes (Kiwada et al 1985b; Baillie et al 1986). However, none of these non-ionic surfactant formulations is likely to survive chronic toxicity research into human intravenous application. Therefore, the topical route of administration seems more appropriate for the use of these formulations, especially as non-ionic surfactants are known to have a penetration-enhancing effect on the skin (Ashton et al 1986; Walters et al 1988; Walters 1989; Kadir et al 1992.

This paper will focus on NSVs intended for dermal or transdermal application of drugs. In previous studies the vesicles were characterized by means of small angle X-ray scattering, freeze fracture electron microscopy, and dynamic light scattering (Hofland et al 1988, 1989). In order to validate the possibilities of the use of NSVs for pharmaceutical application, the safety of these systems should be established. This study involves two different toxicity models. The first, comprising the measurement of the ciliary beat frequency (CBF), which is a tool to measure the safety of intranasally applied drug formulations, was used as a quick and relatively simple screening method. The second, comprising the measurement of cell proliferation of human keratinocytes, which is a method to assess skin irritancy, was more laborious to perform but offers a more relevant model to determine the safety of topically applied drug formulations. Preliminary results have already been published (Hofland et al 1989, 1991). The vesicles used in this study consisted of polyoxyethylene alkyl ethers (C_nEO_m) in which the number of C atoms (n) varied between 12 and 18 and the number of oxyethylene units (m) between 3 and 7. This series of surfactants was chosen in order to be able to vary the physicochemical properties of the vesicles in a systematic way. The physicochemical properties of the vesicles were described in terms of their hydrophilic-lipophilic balance (HLB) value, and their critical micelle concentrations (CMC), and the bilayer rigidity as determined by the gel-liquid transition temperatures and the cholesterol content of

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the vesicles. In addition, the effect of the bond by which the alkyl chain is linked to the polyoxyethylene headgroup on the cell proliferation was investigated. Vesicles were prepared from both oleyl-EO_5 ether and oleyl-EO_5 ester.

The aim of this study was to assess the relationship between the physicochemical properties of the vesicles and the safety of these systems for topical drug administration. Results obtained using the two toxicity models are compared and discussed in this article.

Materials and Methods

Materials

Technical grade non-ionic surfactants of the polyoxyethylene alkyl ether type (C_nEO_m; n = 12, 14, 16 or 18 and m = 3 or 7) were purchased from Servo (Delden, The Netherlands). Gel chromatography studies revealed that in each case the purity of the surfactants was higher than 90% (unpublished data). Cholesterol was obtained from J. T. Baker Chemicals B. V. (Deventer, The Netherlands).

Preparation of non-ionic surfactant vesicles

Small unilamellar vesicles were obtained using the sonication method described by Baillie et al (1985). Large multilamellar vesicles were prepared by heating the small unilamellar NSV suspensions to 80°C for 5 min (Hofland et al 1989). In these studies only large multilamellar vesicles were used. The z-average diameter, determined using photon correlation spectroscopy, ranged from 0·9 to 1·2 μm (Hofland et al 1991). For studying the effect of the alkyl chain length (C_{12} up to C_{18}) and the number of oxyethylene units (EO_3 vs EO_7) on the safety of the surfactants in the vesicular state, it was necessary to add a certain amount of cholesterol in order to be able to prepare vesicles of all surfactants in our series (Israelachvili 1985; Hofland et al 1988). The amounts of surfactant and cholesterol used are given as percentages of the total molar concentration, 30 mM. Sample volumes of 5 mL were used. It was essential to prepare the vesicles at a temperature above the gel-liquid transition temperature of the surfactants; therefore, all vesicle preparations were carried out at 80°C.

The aqueous phases in which the NSVs were prepared consisted of the following media: for studying the NSVs with the intranasal toxicity model the vesicles were prepared in Locke-Ringer buffer (LR) containing 7·72 g NaCl, 0·42 g KCl, 0·16 g $CaCl_2 \cdot 2H_2O$, 1 g dextrose and 0·15 g $NaHCO_3$ dissolved in 1 L water. The pH was adjusted to 7·4. NSVs used for the skin irritancy model were prepared in tissue culture medium consisting of a mixture of Dulbecco's modification of Eagle's Essential Medium (DMEM) and Ham's F12 (3:1) obtained from Flow Laboratories Ltd (Irvine, UK) supplemented with 1 μM isoprenaline, 5% Hyclone calf serum (Greiner GmbH, Pleidelshein, Germany) and 1 μM hydrocortisone. Both the CMCs and the gel-liquid transition temperatures of the surfactants were determined in a phosphate buffered saline solution (PBS) consisting of (mM): 139 NaCl, 2·5 KCl, 8 Na_2HPO_4 and 1·5 KH_2PO_4, adjusted to pH 7·4.

Toxicity model for intranasal application

The safety of intranasally applied formulations was determined by measuring the effect of these formulations on the CBF. CBF measurements were performed on ciliated tissue of chicken embryo trachea with a photo-electric device as described by van de Donk et al (1980).

Inseminated chicken eggs were incubated in a breeding machine at a temperature of 37°C for 19 days. The relative humidity was maintained between 40 and 70%. After decapitation of the embryo the trachea was removed, rinsed with LR and sliced into rings of approximately 1 mm thickness. Hereafter the tissue samples were allowed to stabilize at 31°C for at least one hour to minimize artifacts caused by stress of the tissue. The experiments were performed at 31°C, the physiological temperature of the human nasal mucosa (Duchateau et al 1985). After adding the NSV suspension the CBF was determined every 10 min for 60 min. The CBF at t = 0 was defined as 100%. The CBF of the control tracheae were measured in LR without NSVs. Each experimental formulation contained 60% surfactant and 40% cholesterol in LR at a final concentration of 0·3 mM.

Toxicity model for topical application

The dermal toxicity of NSVs was established by determining the influence of the vesicles on the proliferation of cultured human keratinocytes. The inhibition of the proliferation of SV40 transformed keratinocytes (SVK14) was assayed by measuring the active uptake of neutral red (Borenfreund & Puerner 1985). The vesicles were prepared from 40% cholesterol and 60% surfactant. The surfactants $C_{12}EO_3$, $C_{18}EO_3$, $C_{12}EO_7$, $C_{18}EO_7$ were used. Furthermore NSVs prepared from both oleyl-EO_5 ether and oleyl-EO_5 ester were investigated. An increase in cholesterol content of the bilayers resulted in a decrease in the release rate of encapsulated material and therefore an increase of the rigidity of the bilayers was obtained (Szoka & Papahadjopoulos 1980; Khand et al 1987). The effect of the rigidity of the vesicle bilayers on the cell proliferation was determined by applying liquid state vesicles containing various amounts of cholesterol. Vesicles were prepared from $C_{12}EO_3$, and the amount of cholesterol was increased from 0 up to 40%. The amount of surfactant was kept constant in the initial formulations.

The experiments were carried out as follows: SVK14 cells (Taylor-Papadimitriou et al 1982) were cultured in a mixture of DMEM and Ham's F12 (3:1) medium supplemented with 1 μM isoprenaline at 37°C in humidified atmosphere containing 7% CO_2, as described by Ponec et al (1985). On day zero, 100 μL of cell suspension containing approximately 5000 SVK14 cells was plated to each well of a 96 well cluster plate (Greiner, Pledelshein, Germany). On day one, 100 μL medium containing increasing concentrations of a NSV suspension was added to each well. On day four, 50 μL of 0·05% neutral red (Gurr) solution in 0·9% NaCl was added per well. The neutral red is taken up by the cells by active transport. The cells were reincubated for 3 h at 37°C in an atmosphere containing 7% CO_2. Subsequently, after washing with physiological saline, the cells were lysed by addition of 100 μL of a solution containing 50% ethanol in 0·05 M NaH_2PO_4 per well (Borenfreund & Puerner 1985). After vigorous shaking, the absorption was measured at 550 nm using a Titertek Multiscan. All experiments were performed in octuplicate. The amount of neutral red taken up by the control cells, incubated with tissue culture medium in the

absence of the test agent, was defined as 100% proliferation. The IC50 is defined as the concentration of the test agent that inhibits 50% of the cell proliferation at day four.

Physicochemical properties of the vesicles

Hydrophilic-lipophilic balance (HLB). Griffin (1955) introduced the HLB as a tool to classify surfactants. In one approach this parameter is defined as the weight-ratio of the hydrophilic and lipophilic moiety of an amphiphilic molecule, on a scale from 1–20 ($20 \times MW_{hydrophilic}/MW_{total}$).

Critical micelle concentration (CMC). The surface tension at increasing surfactant concentrations in PBS was determined by the stalagmometer method. The number of drops of a 5 mL sample was assessed. The surface tension was calculated using the following equation: $\gamma_s = (\gamma_w \cdot N_w)/N_g$; where γ is the surface tension, N is the number of drops, w signifies water and s signifies surfactant solution. The surface tension of the surfactant solution decreases with increasing surfactant concentrations as long as no micelles are formed, while the surface tension remains constant after micelle formation. Plotting the surface tension against the surfactant concentration, the CMC was defined as the intersection of the decreasing curve and the plateau value.

Gel-liquid transition temperatures. The gel-liquid transition temperatures of the NSV suspensions were determined by high sensitivity differential scanning calorimetry (HSDSC). The HSDSC curves were determined for those formulations

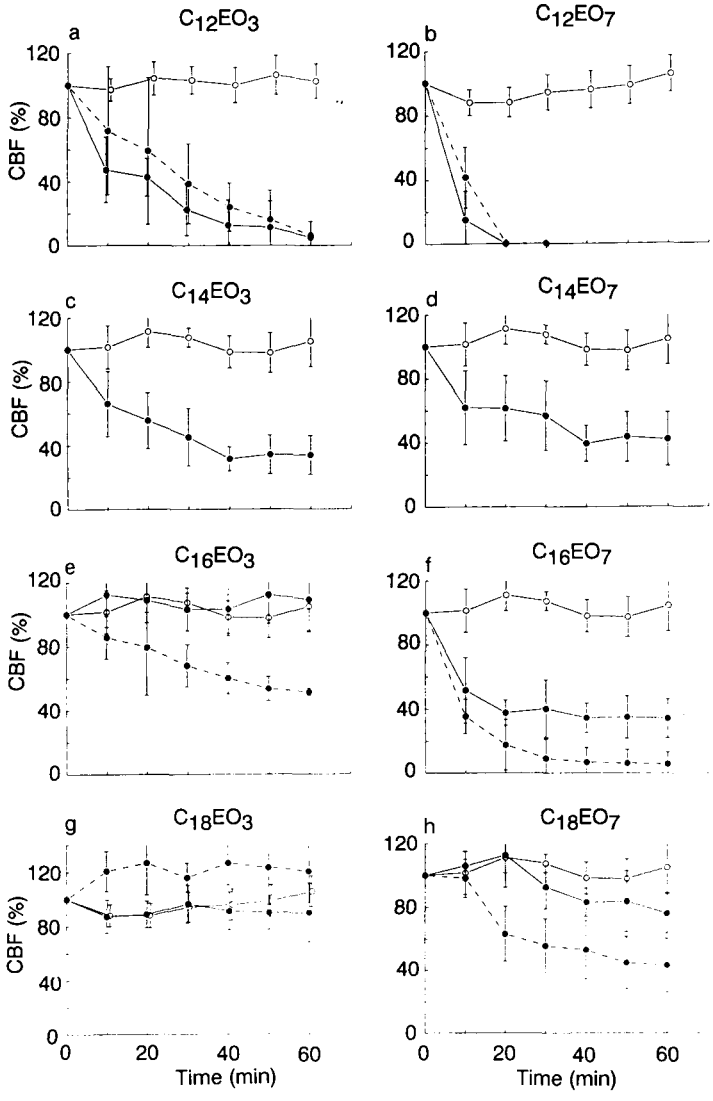

FIG. 1. In the graphs a–h the effect of NSVs on the ciliary beat frequency (CBF) was plotted as a function of the exposure time. The NSV formulations consisted of 60% surfactant and 40% cholesterol (0·3 mM) in Locke-Ringer buffer (LR). On the left hand side (graphs a, c, e, and g) the effect of formulations containing a polyoxyethylene headgroup of 3 units on the CBF is plotted. On the right hand side (graphs b, d, f, and h) the effects of EO_7 formulations are given. In the graphs a/b, c/d, e/f, and g/h the length of the alkyl chains was varied, containing 12, 14, 16, and 18 C atoms, respectively. Data are mean ± s.d. of 6 experiments. o Samples containing NSVs, ● CBFs measured in pure LR. The experiments were performed at 31 (——) and 37°C (– – –).

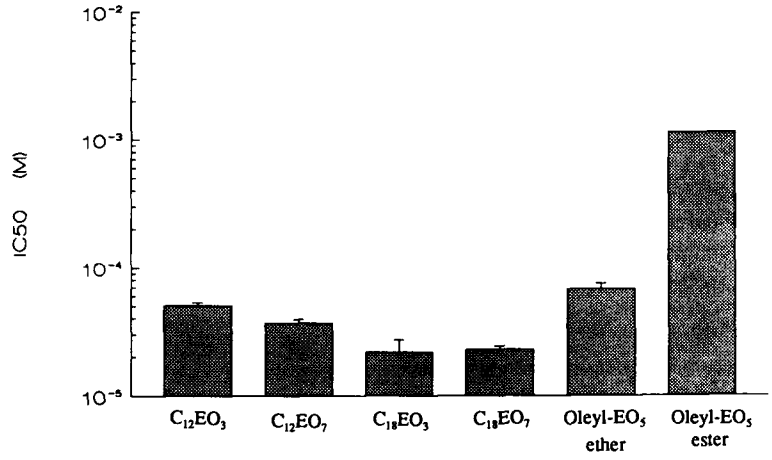

FIG. 2. The effect of the alkyl chain length (C_{12} vs C_{18}) and the number of oxyethylene units (EO_3 vs EO_7) as well as the type of bonds by which the alkyl chain is linked to the polyoxyethylene headgroup (either an ether or an ester) on the cell proliferation of transformed keratinocytes (SVK14). As a measure of the toxicity the decrease of the active uptake of neutral red was determined by measuring the extinction at 550 nm. The amount of neutral red taken up by the control cells (incubated with tissue-culture medium) was defined as 100% proliferation. IC50 is defined as the concentration that inhibits 50% of the cell proliferation.

that were in gel state at room temperature (20°C): $C_{16}EO_3$, $C_{16}EO_7$, $C_{18}EO_3$ and $C_{18}EO_7$. Both the HSDSC curves of 100% surfactant and 60% surfactant/40% cholesterol were determined. The calorimetric experiments were conducted in a Microcal MC-2 microcalorimeter (Microcal Amherst, MA, USA), interfaced with an IBM PC 32 personal computer. The calorimetric run was obtained under excess nitrogen pressure (2 atm). The experiment was conducted at a scan rate of 60 K h^{-1}, over a temperature range of 5 to 70°C. The gel-liquid transition temperature of a formulation was defined as the onset of the transition, which was defined as the temperature where the trace moves off the baseline.

Results

Toxicity model for intranasal application

The effect of 8 different NSV formulations on the CBF of chicken trachea in-vitro is given in Fig. 1. $C_{16}EO_3$, $C_{18}EO_3$ and $C_{18}EO_7$ formulations do not remarkably influence the CBF at 31°C. The preparations $C_{14}EO_3$, $C_{14}EO_7$ and $C_{16}EO_7$ reduce the CBF values to 35–40% of their initial frequencies after 60 min exposure at 31°C; $C_{12}EO_3$ almost totally inhibits the ciliary movement within 60 min, whereas $C_{12}EO_7$ induces ciliostasis within 20 min. When after 60 min exposure to the NSV suspension the tracheal tissue was replaced in pure LR, the CBF values returned to normal within 5 min for all NSVs studied, except for $C_{12}EO_7$, the effect of which appeared to be irreversible. It is apparent that both the acyl chain length and the number of the polyoxyethylene units in the headgroup influence the effect on the CBF. The longer the alkyl chain of the surfactant the less the inhibitory effect of the vesicular suspension on the ciliary movement. The opposite correlation was observed for the length of the headgroup.

Toxicity model for topical application

Fig. 3 displays typical inhibition profiles. There appeared to be a strong correlation between the surfactant concentration and the effect on the % cell proliferation, which is not specific for $C_{12}EO_3$ formulations, but was found for all the surfactant formulations used in this study. Therefore, it was possible to determine the IC50 accurately. As apparent from Fig. 2 the IC50 values of the C_{12} and C_{18} vesicles show only minor differences, indicating that C_{12} formulations might be safer to use than the C_{18} preparations. The difference in the number of oxyethylene units has no significant effect on the cell proliferation.

The influence of NSVs prepared from either oleyl-EO_5 ether or the oleyl-EO_5 ester on the tissue culture are also shown in Fig. 2. The concentration of the ether-surfactant that inhibited cell proliferation by 50% was about one-sixteenth that of the ester-surfactant.

The inhibition of cell proliferation as a function of the $C_{12}EO_3$/cholesterol concentration of NSVs prepared from various surfactant/cholesterol ratios, is plotted in Fig. 3; the

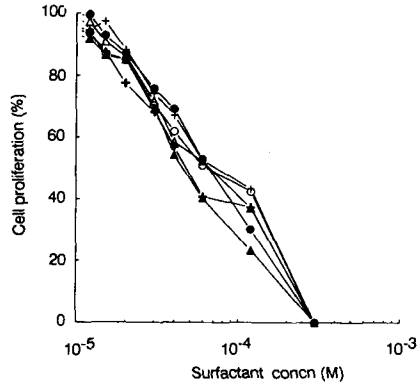

FIG. 3. The effect of cholesterol, incorporated in the bilayers of liquid state $C_{12}EO_3$ NSVS, on the cell proliferation of transformed keratinocytes (SVK14), As a measure of the toxicity the decrease of the active uptake of neutral red was determined. The amount of neutral red taken up by the control cells is defined as the 100% reference value. +, 0%; △, 1%; ○, 5%; +, 10%; ▲, 20%; and ●, 40% cholesterol.

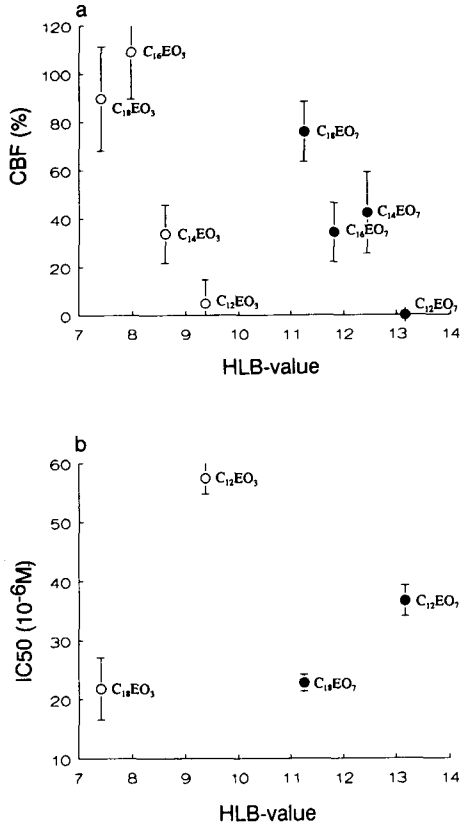

FIG. 4. a. The % CBF, remaining after 1 h exposure to the NSV suspension consisting of 60% surfactant and 40% cholesterol (0·3 mM) in Locke-Ringer buffer at 31°C, plotted against the hydrophilic lipophilic balance (HLB) of the non-ionic surfactants of NSVs. ○, EO_3 surfactants; ●, EO_7 surfactants. b. The IC50, obtained after four days exposure to the NSV suspension, plotted against the HLB of the non-ionic surfactants of NSVs. ○, EO_3 surfactants; ●, EO_7, surfactants.

cholesterol content in the lipid bilayers and therefore the rigidity of the bilayers does not show a significant effect on the proliferation of the cultured human keratinocytes.

Physicochemical properties of the vesicles

Hydrophilic-lipophilic balance (HLB). The % CBF, remaining after one hour exposure to an NSV suspension as a function of the HLB value of the surfactant, was plotted in Fig. 4a. No relationship was found between the HLB of the surfactants and the effects of the surfactants on the CBF.

The IC50, obtained after four days exposure to an NSV suspension, was plotted as a function of the HLB value of the surfactant (Fig. 4b). Again, no relationship was found between the HLB of the surfactants and the effects of the surfactants on the cell proliferation of human keratinocytes.

Critical micelle concentration (CMC). The CMC values of $C_{12}EO_7$, $C_{14}EO_7$ and $C_{16}EO_7$ were 0·24 (±0·01), 0·45 (±0·05) and 0·85 (±0·05) mM, respectively. The CMCs of the other surfactants could not be determined. Comparing the safety of the surfactants, it was found that in a number of cases a positive correlation exists between the CMC value and the effect on the CBF e.g. between $C_{12}EO_7$ and all of the C_nEO_3 formulations. However, in a number of cases the opposite correlation was observed: e.g. comparing, $C_{12}EO_3$ with $C_{14}EO_7$ and $C_{16}EO_7$ or comparing $C_{16}EO_7$ with $C_{14}EO_7$ and $C_{12}EO_7$. There data indicates that there is no relationship between CMC (i.e. the free surfactant concentration) and the effect on CBF.

The $C_{12}EO_7$ vesicles have a relatively high CMC compared with e.g. $C_{12}EO_3$, $C_{18}EO_7$ and $C_{18}EO_3$. However, there are only minor differences in cytotoxicity of these surfactants, indicating that there is no correlation between the CMC of these surfactants and their effect on cell proliferation.

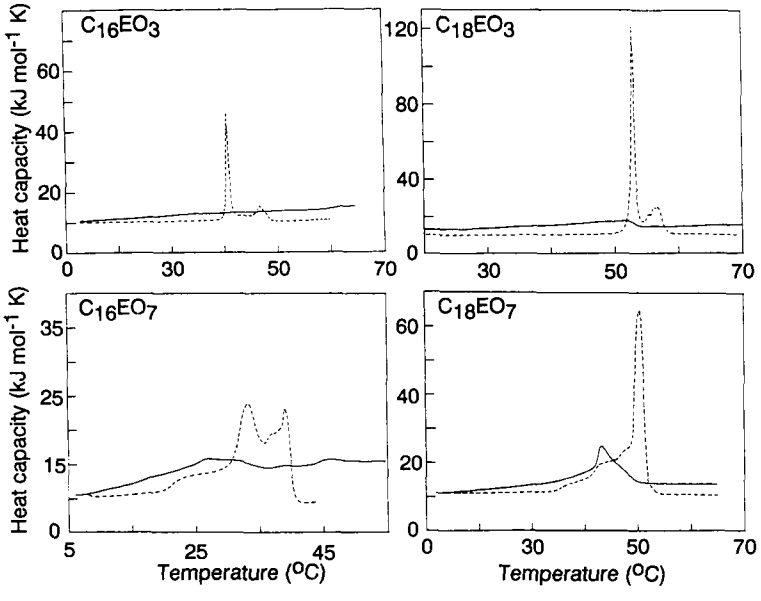

FIG. 5. The DSC curves of 30 mM of 100% surfactant (——) and of 60% surfactant/40% cholesterol (---). The gel-liquid transition temperature of a formulation was defined as the onset of the transition.

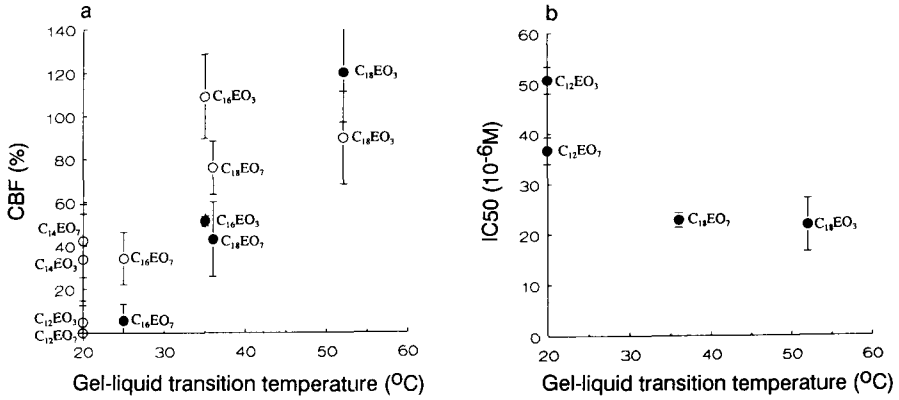

FIG 6. a. The % CBF, remaining after 1 h exposure to the NSV suspension consisting of 60% surfactant and 40% cholesterol (0·3 mM) in Locke-Ringer buffer, plotted against the gel-liquid transition temperatures of the NSV formulations. ○, Results obtained at 31°C; ●, results obtained at 37°C. Experiments at 37°C were only performed for those formulations that had gel-liquid transition temperatures above room temperature: $C_{16}EO_3$, $C_{16}EO_7$, $C_{18}EO_3$, and $C_{18}EO_7$. The % CBF of those formulations having transition temperatures equal or lower than room temperature are given on the y-axis. b. The IC50, obtained after four days exposure to the NSV suspension, plotted against the gel-liquid transition temperatures of the NSV formulations. The IC50 values of those formulations having transition temperatures equal to or lower than room temperatures are given on the y-axis.

Gel-liquid transition temperatures. The NSV suspensions show complicated transitions as presented in Fig. 5. There appeared to be a gel-liquid transition range, and not one well defined gel-liquid transition temperature. However, the gel-liquid transition temperature was defined as the starting point of the transition range. Cholesterol has a decreasing effect on both the gel-liquid transition temperatures and the gel-liquid transition enthalpies (Szoka & Papahadjopoulos 1980). Fig. 5 shows that, in the case of the 100% $C_{16}EO_3$ formulation, sharp transitions occur; however, when 40% cholesterol was added almost no enthalpy change could be detected. Therefore, the transition temperature of the 60% $C_{16}EO_3$/40% cholesterol formulation was extrapolated from gel-liquid transition temperatures obtained from $C_{16}EO_3$ formulations containing 0, 5, 10 and 20% cholesterol (unpublished data). The gel-liquid transition temperatures for the NSV suspensions containing 60% surfactant and 40% cholesterol were found to be 25, 35, 36 and 52°C for $C_{16}EO_7$, $C_{16}EO_3$, $C_{18}EO_7$ and $C_{18}EO_3$, respectively.

The % CBF after one hour exposure to a NSV suspension at 31°C was plotted against the gel-liquid transition temperature of the NSV formulation (Fig. 6a). A correlation appeared to exist between the state of the bilayer and the effect of the vesicles on the CBF. Liquid state vesicles have a significantly greater effect on the reduction of the CBF than gel state vesicles. In order to investigate this phenomenon, CBF measurements were also performed at 37°C. At this temperature $C_{16}EO_3$, $C_{16}EO_7$ and $C_{18}EO_7$ NSVs were in the liquid state. $C_{18}EO_7$ vesicles have a significantly greater inhibitory effect on the CBF at 37 than at 31°C. However, the gel state $C_{18}EO_3$ vesicles exhibit a slight increase in CBF at 37°C. Both the $C_{12}EO_3$ and $C_{12}EO_7$ preparations, being already in liquid state at 31°C, show similar effects on the CBF at 31 and at 37°C. Fig. 1 shows in more detail the effect of the NSV preparation on the CBF at 37°C.

The correlation between the gel-liquid transition temperature of a surfactant and the toxicity of that surfactant was not apparent for the cytotoxicity model (Fig. 6b): the liquid state vesicles ($C_{12}EO_3$ and $C_{12}EO_7$) seem to be safer to use than the one in the gel state, $C_{18}EO_3$. $C_{18}EO_7$ vesicles being in the liquid state at 37°C appeared as equally toxic as the gel state $C_{18}EO_3$ vesicles.

Discussion

The vesicles that were used in this study were large, multilamellar, and polydisperse. This was determined by photon correlation spectroscopy, small angle X-ray diffraction, and freeze fracture electron microscopy (Hofland et al 1991). However, the reproducibility of the vesicle characteristics appeared to be good. New (1990) reported that, especially for small unilamellar liposomes having a diameter of less than 50 nm, vesicle membranes show abnormalities in the packing of the lipids, which can cause a rapid intermembrane transfer of lipid-soluble compounds. The average diameter of the vesicles used in this study ranged from 0·9 to 1·2 nm, while the minimal size of NSVs varied between 100 and 200 nm, as determined by photon correlation spectroscopy. Therefore, the curvature of the NSV bilayers is low and is thus not considered to play an important role in the vesicle toxicity.

The relevance of the in-vitro ciliotoxicity model is based on the fact that the mucociliary clearance is related to the ciliary beat frequency (CBF) in the nose (Duchateau et al 1985). Embryonal chicken trachea has proved to be a good model for human nasal ciliated tissue (van de Donk 1982; van de Donk et al 1982). Therefore, the influence on the CBF of this tissue can be used as a tool to measure possible toxicity of intranasal drug formulations. Changes in the mucociliary clearance can be due to an impairment of ciliary beating, which hampers the self-cleaning capacity of the nose. van de Donk et al (1981) showed that many of the commercially available nasal drops are, to a greater or lesser extent, ciliotoxic. Several products (5 times diluted) were investigated with the ciliotoxicity model. Comparing the present data with those reported previously, it is evident that the

effect of preparations containing short acting decongestants, such as Endrine (Wyeth) and Fenox (Boots), are similar to the effect of $C_{12}EO_3$ vesicles on the CBF. Furthermore, preparations with a prolonged decongestive action such as Otrivin (Ciba) and Sinex (Richardson-Merrell) and antimicrobial agents such as chloramphenicol (Bournonville-Pharma) and rhinamide (Bailly) have been found to show moderate ciliotoxicity, similar to that induced by $C_{14}EO_3$, $C_{14}EO_7$ and $C_{16}EO_7$ formulations. In studies with rabbits and rats, Deurloo et al (1989) found that 15 mM sodium taurodihydrofusidate (STDHF) added to an intranasally applied insulin formulation results in a significant enhancement of insulin absorption through the nasal mucosa. The effect of this penetration enhancer on the CBF of embryonal chicken trachea was investigated by Hermens et al (1990), who found that 15 mM STDHF induces a complete ciliostasis within 10 min. This effect is comparable with our findings with $C_{12}EO_7$ NSVs. The addition of 3 mM STDHF had the same effect on the CBF as $C_{12}EO_3$ formulations. Products having a mild and reversible effect on the CBF are acceptable to be used in drug formulations. The ciliotoxicity model was used to screen NSV formulations for safety and to compare the results of a series of NSV formulations. The results were compared with those obtained in a different toxicity model used to evaluate the safety of formulations applied topically.

The safety of NSVs used for topical administration of drugs was studied by determining the influence of NSVs on the proliferation of cultured human keratinocytes. The relevance of this in-vitro cytotoxicity model is based on the fact that the skin irritancy of compounds is often related to a direct interaction between the compound and viable skin cells. A relationship between in-vitro toxicity to keratinocytes and in-vivo skin irritancy is assumed. Recently the cytotoxicity of a series of *N*-alkyl-aza-cycloheptane-2-ones has been investigated (Ponec et al 1989). In that study the alkyl chain length was varied between 2 and 16 C atoms. At alkyl chain lengths between 2 and 8 C atoms, toxicity was found to increase as the length of the alkyl chain increased. No significant differences in cytotoxicity could be found for the alkyl chain lengths between 8 and 16 C atoms. Although in our experiments different compounds were used, the results were similar: a similar concentration dependence was observed, and it was shown that by varying the alkyl chain length from 12 to 18 no important differences could be found. Moreover, the C_{12} compound, Azone, and the C_{12} NSVs appeared to have similar IC50 values: 5×10^{-5} M. The observation that ester-type surfactants, in which the alkyl chain is linked to the polyoxyethylene headgroup by an ester bond, are less toxic than ether-type surfactants, has also been made in experiments, in which surfactants were tested in combination with propylene glycol (Kadir et al 1992). The IC50 of the ester was ten times higher than that of the ether compound. Ester bonds may be easily hydrolysed by enzymes of the keratinocytes. Therefore, the lower toxicity of the ester surfactants may be explained by their chemical instability. It is remarkable that the oleyl-EO_5 ether and ester surfactants in the vesicular form appeared to be much safer than the same compounds dissolved in propylene glycol. The IC50 values of the vesicular forms were $6{\cdot}7 \times 10^{-5}$ and $1{\cdot}1 \times 10^{-3}$ M for the ether and ester, respectively. Kadir et al (1992) recorded IC50 values of $1{\cdot}2 \times 10^{-5}$ and $1{\cdot}2 \times 10^{-4}$ M.

In contrast to the ciliotoxicity results, the physicochemical properties of the surfactants do not seem to have an important effect on the cell proliferation of cultured human keratinocytes. These discrepancies may be due to differences in vesicle-cell interactions between the two toxicity models: e.g. in the case of the ciliotoxicity model the ciliated cells are present in a confluent cell layer, whereas the human keratinocytes are cultured in a sparse density. Moreover, the duration of interactions between the NSV suspensions and cells in the ciliotoxicity studies was only 1 h, while the keratinocytes were exposed to the NSVs for three days. Therefore, the kinetic aspects of the effect of surfactants on the ciliated tissue may play an important role which is not observed in the tissue culture model. In spite of the difficulty in comparing the results obtained in the two toxicity models, the results obtained from both methods, give an important insight into the factors which may play a role in the safety of the NSV suspensions.

A relationship between the HLB and the penetration enhancing effect of non-ionic surfactants of the polyoxyethylene alkyl ether type on intranasally applied insulin in rats was demonstrated by Hirai et al (1981a, b). There was a 6-fold increase in the bioavailability of insulin for all surfactants that had HLB values ranging from 8 to 14. The surfactants used in this study have HLB values between 8 and 14. On the basis of our results it was not possible to find any relationship between the HLB and the effects on CBF, in-vitro. Combining our CBF results with those of Hirai et al (1981a, b), it can be concluded that although surfactants with HLB values between 8 and 14 induce the same penetration enhancement, this does not necessarily mean they are equally safe to use.

The toxic effects of surfactant formulations are often thought to be related to the amount of free surfactant present. The CMC of a surfactant is a measure of the amount of free surfactant. For this reason, the toxicity of the NSV suspensions may be expected to be related to the CMC of the surfactants used. However, such a relationship was not found, either in the ciliotoxicity model or in the cytotoxicity model. It may therefore be concluded that the amount of free surfactant in the NSV suspension is not primarily responsible for the toxic effects of the formulation. Furthermore, it appeared that the presence of micelles does not influence the toxicity, since the ciliotoxicity of the liquid state vesicles $C_{12}EO_3$ and $C_{14}EO_3$ does not significantly differ from that of $C_{12}EO_7$ and $C_{14}EO_7$ formulations, respectively, at 31°C. In the case of the $C_{12}EO_3$ and $C_{14}EO_3$ surfactants no micelles can be formed, in contrast to the $C_{12}EO_7$ and $C_{14}EO_7$ formulations.

The mobility of the surfactants in a formulation may affect the ciliotoxicity of that formulation. The mobility of the surfactant molecules in an NSV suspension is determined by the rigidity of the bilayers. Gel state bilayers are less toxic than liquid bilayers because the surfactant molecules that are present in the liquid bilayers can be readily exchanged with the environment. Therefore, they can be more easily incorporated in the membranes of target cells, leading to more severe effects on biological tissues. The combination of results obtained by DSC and CBF experiments confirm the view that, concerning the ciliotoxicity, liquid state vesicles are more toxic than gel state vesicles. However, although

cholesterol is known to decrease the mobility of molecules in the bilayers of liquid state vesicles, in our studies it did not appear to have an effect on the cell proliferation.

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References

Ashton, P., Hadgraft, J., Walters, K. A. (1986) Effects of surfactants in percutaneous absorption. Pharm. Acta. Helv. 61, 8: 228–235

Baillie, A. J., Florence, A. T., Hume, L. R., Muirhead, G. T., Rogerson, A. (1985) The preparation and properties of niosomes—non-ionic surfactant vesicles. J. Pharm. Pharmacol. 37: 863–868

Baillie, A. J., Coombs, G. H., Dolan, T. F., Laurie, J. (1986) Non-ionic surfactants vesicles, niosomes, as a delivery system for the anti-leishmanial drug, sodium stibogluconate. Ibid. 38: 502–505

Borenfreund, E., Puerner, J. A. (1985) Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicol. Lett. 24:119–124

Deurloo, M. J. M., Hermens, W. A. J. J., Romeyn, S. G., Verhoef, J. C., Merkus, F. W. H. M. (1989) Absorption enhancement of intranasally administered insulin by sodium taurodihydrofusidate (STDHF) in rabbits and rats. Pharm. Res. 10: 853–856

Duchateau, G. S. M. J. E., Graaimans, K., Zuidema, J., Merkus, F. W. H. M. (1985) Correlation between nasal ciliary beat frequency and mucus transport rate in volunteers. Laryngoscope 95: 854–859

Echegoyen, L. E., Hernandez, J. C., Kaifer, A. E., Gokel, G. W., Echegoyen, L. (1988) Aggregation of steroidal ethers: the first example of nonionic liposomes (niosomes) formed from neutral crown ether compounds. J. Chem. Soc. Chem. Comm. 12: 836–837

Griffin, W. C. (1955) Calculation of "HLB" values of nonionic surfactants. Am. Perfumer Essent. Oil Rev. 65: 26–29

Handjani-Vila, R. M., Ribier, A., Rondot, B. A., Vanlerberghie, G. (1979) Dispersions of lamellar phases of non-ionic lipids in cosmetic products. Int. J. Cosm. Sci. 1: 303–314

Hermens, W. A. J. J., Hooymans, P. M., Verhoef, C., Merkus, F. W. H. M. (1990) Effects of absorption enhancers on human nasal tissue ciliary movement in vitro. Pharm. Res. 144–146

Hirai, S., Yashiki, T., Mima, H. (1981a) Effect of surfactants on the nasal absorption of insulin in rats. Int. J. Pharm. 9: 165–172

Hirai, S., Yashiki, T., Mima, H. (1981b) Mechanisms for the enhancement of the nasal absorption of insulin by surfactants. Ibid. 9: 173–184

Hofland, H. E. J., Bouwstra, J. A., Talsma, H., Junginger, H. E. (1988) Characterization of non-ionic surfactant vesicles: structure, size and stability. Proceed. Intern. Symp. Control. Rel. Bioact. Mater. 15: 406–407

Hofland, H. E. J., Bouwstra, J. A., Verhoef, J., Junginger, H. E. (1989) Niosomes: a study of structure, stability, drug release and toxicological aspects. Ibid. 16: 136–137

Hofland, H. E. J., Bouwstra, J. A., Spies, F., Junginger, H. E. (1990) The interactions between non-ionic surfactant vesicles and human skin in vitro. Ibid. 17: 39–40

Hofland, H. E. J., Bouwstra, J. A., Punec, M., Bodde, H. E., Spies, F., Verhoef, H., Junginger, H. E. (1991) Interactions of non-ionic surfactant vesicles with cultured ketatinocyter and human skin in-vitro; a survey of toxicological aspects and ultrastructural changes in stratum corneum. J. Control. Rel. 16: 155–168

Israelachvili, J. N. (1985) Thermodynamic and geometric aspects of amphiphile aggregation into micelles, vesicles and bilayers, and the interactions between them. Proceed. Int. School Phys. 38–58

Kadir, R., Tiemessen, H. L. G. M., Boddé, H. E., Ponec, M., Junginger, H. E. (1992) Effects of oleyl surfactants on nitroglycerin permeation through isolated human stratum corneum and their toxicity to cultured skin cells. J. Control. Rel. In press

Khand, L., Rogerson, A., Halbert, G. W., Baillie, A. J., Florence, A. T. (1987) The effect of cholesterol on the release of doxorubicin from non-ionic surfactant vesicles (niosomes). J. Pharm. Pharmacol. 39 (Suppl.): 41P

Kiwada, H., Niimura, H., Kato, Y. (1985a) Tissue distribution and pharmacokinetic evaluation of the targeting efficiency of synthetic alkyl glycoside vesicles. Chem. Pharm. Bull. 33: 2475–2482

Kiwada, H., Niimura, H., Fujisaki Y., Yamada, S., Kao, Y. (1985b) Application of synthetic alkyl glycoside vesicles as drug carriers. 1. Preparation and physical properties. Ibid. 33: 753–759

New, R.C.C. (1990) Liposomes: a practical approach. In: Richwood, D., Hames, B.D. (eds) Practical Approach Series. IRL Press, Oxford University

Ozer, A. Y., Hincal, A. A., Bouwstra, J. A. (1991) A novel drug delivery system: non-ionic surfactant vesicles (niosomes). Eur J. Pharm. Biopharm. 37: 75–79

Ponec, M., Lavrijsen, S., Kempenaar, J., Havekes, L., Boonstra, J. (1985) SV40 transformed (SVK14) and normal keratinocytes: similarity in the expression of low density lipoprotein, epidermal growth factor, glucocorticoid receptors, and the regulation of lipid metabolism. J. Inv. Derm. 85: 476–482

Ponec, M., Haverkort, M., Soei, Y. L., Kempenaar, J., Brussee, J., Bodde, H. E. (1989) Toxicity screening of n-alkylazacycloheptan-2-one derivatives in cultured human skin cells: structure-toxicity relationships. J. Pharm. Sci. 78: 738–741

Szoka, F. C., Papahadjopoulos, D. (1980) Comparative properties and methods of preparation of lipid vesicles (liposomes). Ann. Rev. Biophys. Bioeng. 9: 467–508

Taylor-Papadimitriou, J., Purkis, P., Lane, E. B., McKay, I. A., Chang, S. E. (1982) Effects of SV40 transformation of the cyto skeleton and behavioural properties of human keratinocytes. Cell Differ. 11: 169–180

van de Donk, H. J. M. (1982) Correlation between the sensitivity of the ciliary beat frequency of human adenoid tissue and chicken embryo tracheus for some drugs. Thesis, University of Amsterdam, The Netherlands. Nasal Medication and Ciliary Movement. Chapter IV

van de Donk, H. J. M., Zuidema, J., Merkus, F. W. H. M. (1980) The influence of the pH and osmotic pressure upon tracheal ciliary beat frequency as determined with a new photo-electric registration device. Rhinology 18: 93–104

van de Donk, H. J. M., Zuidema, J., Merkus, F. W. H. M. (1981) The effects of nasal drops on the ciliary beat frequency of chicken embryo tracheas. Ibid. 19: 215–230

van de Donk, H. J. M., Zuidema, J., Merkus, F. W. H. M. (1982) Correlation between the sensitivity of the ciliary beat frequency of human adenoid tissue and chicken embryo tracheas for some drugs. Thesis, University of Amsterdam, The Netherlands. Nasal Medication and Ciliary Movement. Chapter IV

Walters, K. A., Walker, M., Olejnik, O. (1988) Non-ionic surfactants effects on hairless mouse skin permeability characteristics. J. Pharm. Pharmacol. 40: 525–529

Walters, K. A. (1989) Transdermal Drug Delivery, Dekker, New York, Basel, pp 1–22