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Research paper

Effects of ethanol/L-menthol on the dynamics and partitioning of spin-labeled lipids in the stratum corneum

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Abstract

The interaction of ethanol as well as ethanol/L-menthol mixtures with the uppermost layer of epidermis, the stratum corneum, was investigated by electron paramagnetic resonance (EPR) spectroscopy utilizing spin-labeled analogs of androstanol (ASL), stearic acid (5-DSA) and methyl stearate (5-DMS). The EPR spectra of these spin probes structured in stratum corneum tissue of neonatal rat are characterized by the coexistence of two spectral components indicating the presence of two classes of spin labels with very different states of mobility. Probably, one class of spin labels is H-bonded to the polar surface of the membrane and another class corresponds to spin labels more deeply inserted in the hydrophobic core. EPR results showed that in the ethanol range 0–70% neither fluidity in stratum corneum membranes nor the relative fractions of these two components changes were observed. Instead, ethanol only caused a selective extraction of spin labels. The removal of the steroid ASL began at 30% ethanol, reaching extraction levels over 50% at ethanol concentrations of 60–70%, whereas the more hydrophobic 5-DMS was partially removed only with 70% ethanol. Addition of 5% L-menthol to the solvent containing 20% ethanol increases both the mobility and the fraction of those spin labels situated in the hydrophobic core (more mobile spectral component). Altogether, these findings suggest that the L-menthol stabilizes mainly in the central region of stratum corneum membranes attracting the membrane lipids and causing hydrogen bond ruptures in the polar membrane interface.
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Keywords: Stratum corneum; EPR; Spin label; L-Menthol; Ethanol

1. Introduction

The topical administration of drugs has proven to be a suitable alternative to the oral route due to the low metabolic activity of skin as compared to that found in the gastro-intestinal tract and liver. Another advantage of this route is associated to the possibility to obtain a continuous delivery profile, which is required for a variety of drugs with systemic activity. However, the effectiveness of the transdermal drug delivery system depends on the drug capacity in penetrating the skin at a sufficient concentration in order to achieve therapeutical levels. The main permeability barrier for exogenous chemical absorption is the stratum corneum (SC), the outermost skin layer of about 10–20 μm thickness, composed of corneocytes and a matrix of lamellar lipids. Corneocytes are flat anucleated squamous cells containing an insoluble cell envelope of cross-linked proteins and a cell lipid envelope composed mainly of ω-hydroxyceramides covalently bound to the periphery of the cell envelope [1–3]. The intercellular region contains a complex lipid mixture in continuity with the cell lipid envelope, which self-assembles into an ordered multilayer

Abbreviations: SC, stratum corneum; 5-DSA, 5-doxyl stearic acid; 5-DMS, 5-doxyl methyl stearate; ASL, 17β-hydroxy-4′,4′-dimethylspiro(5α-androstane-3,2′-oxazolidin)-3′-yloxyl; EPR, electron paramagnetic resonance; NLLS, nonlinear least-squares fitting program.

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structure known as lipid lamellae, consisting mainly of ceramides (40–50%), free fatty acids (15–25%), cholesterol (20–25%) and cholesteryl sulfate (5–10%) [4].

Chemical enhancers have been recognized as an efficient approach to increase percutaneous absorption, which reversibly reduces the barrier function of the SC [5]. It is generally accepted that penetration enhancers may increase the permeability of a drug by affecting the intercellular lipids of the SC via extraction or fluidization [6] and/or by increasing the partitioning of the drug into the SC [7]. Beyond that, some chemicals cause conformation changes in the keratinized protein component, increasing the permeability of drugs through the intracellular SC paths [5]. Previous works have revealed that organic solvents such as ethanol in water present a suitable vehicle combination for transdermal permeation of zidovudine (AZT), a hydrophilic drug with low diffusion through the intercellular SC lipids [8]. Nevertheless, the permeation rate achieved with this solvent system was only modest. Recently, inclusion of the terpenes 1,8-cineole and L-menthol at 5% w/v in a formulation containing ethanol 66.6% showed an enhancement effect on percutaneous AZT absorption through rat skin, which would be sufficient to reach therapeutically effective blood concentrations [9,10]. Terpenes are volatile and fragrant substances constituent of essential oils, which contain only carbon, hydrogen and oxygen atoms and are commonly used in flavorings, perfumes, and medicines. The synergistic penetration enhancement effect of ethanol and terpenes is well documented [9–11].

The aim of this study was to investigate the effect of ethanol and ethanol/L-menthol mixtures in the intercellular matrix lipids of SC using electron paramagnetic resonance (EPR) spectroscopy. The line-shape EPR spectrum of spin-labeled fatty acids is highly sensitive to motional properties of probes, permitting to monitor in detail the changes that occur in the lipid bilayers of SC due to addition of drugs [12–16]. Moreover, EPR spectroscopy has been employed as an efficient tool to distinguish the extraction or fluidization effect of a particular penetration enhancer. A recent report [13] showed that the permeation enhancer 1-methyl-2-pyrrolidone acts as an extractor rather than a fluidizer of the SC lipid membranes. In our investigation, three spin labels with different hydrophobicity degree were utilized in order to obtain more insights into the ethanol and ethanol/menthol effects on the biophysical properties of the SC membranes.

2. Materials and methods

2.1. Preparation of SC membranes

SC membranes of newborn Wistar rats less than 24 h old were prepared as described previously [12,13]. After the animals were killed, their skin was excised and fat removed by rubbing in distilled water. The skin was allowed to stand for 5 min in a desiccator containing 0.5 L of anhydrous ammonium hydroxide, after which it was floated in distilled water with the epidermal side in contact with the water for 2 h. The SC was then removed, placed on filter paper and transferred to a Teflon-coated screen. Subsequently, it was washed with distilled water and allowed to dry at room temperature. Finally, the membranes were stored with 1 L of silica gel in a desiccator under a moderate vacuum.

2.2. Spin labeling and treatment of Intact SC

The three spin labels: 5-DSA, having the nitroxide radical moiety (doxyl) in the 5th carbon atom of the acyl chain, the methylated derivative 5-DMS and the steroid androstane (ASL) (Fig. 1) were purchased from Sigma Chem. Co. (St. Louis, MO). In order to prevent nitroxide reductions at high ethanol concentrations, the sulfhydryl group of the SC tissue was blocked incubating the SC membranes in a solution of 50 mM *N*-ethyl maleimide (NEM) (Sigma Chem. Co., St. Louis, MO) for about 15 h. A small aliquot (1 μL) of stock solution of spin label in ethanol (10 mM) was placed on a glass plate and, after the solvent evaporated, the SC membrane (3 mg) was suspended with 50 μL of acetate-buffered saline (10 mM acetate, 150 mM NaCl and 1 mM EDTA, pH 5.1) on the same site where the spin label was placed and gently mixed with a small stick for ~10 min. After spin labeling, all the SC membranes were incubated for 2 h in buffer–ethanol mixtures. Using a microsyringe piston the intact SC membrane was then introduced into a capillary tube for EPR measurements. After the EPR measurements the SC samples were removed from the capillary and immediately weighed to determine the solvent content. A stock solution of L-menthol (Acros organics, New Jersey, USA) in ethanol was prepared and the appropriate concentration of terpene was obtained by dilution of this stock in the acetate buffer containing 20% ethanol. The SC membranes (3 mg) were incubated with 50 μL of solvent for 3 h.

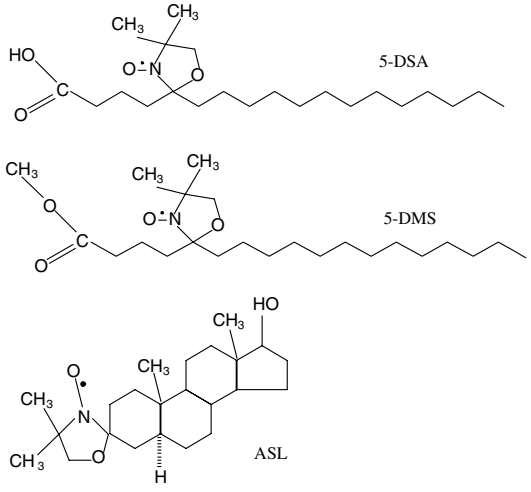

Fig. 1. Chemical structures of the spin labels used in this work.

2.3. EPR spectroscopy

A Bruker ESP 300 spectrometer equipped with an ER 4102 ST resonator and operating in the X-band (9.4 GHz) was utilized in these investigations. The operation conditions of the equipment were: microwave power of 20 mW; modulation frequency of 100 KHz; modulation amplitude of 0.6–1.0 G; magnetic field scan of 100 G; sweep time of 168 s; and detector time constant of 41 ms. The temperature was controlled within 0.3 °C by a nitrogen stream system (Bruker, Rheinstetten, Germany). EPR spectra simulations were performed using the NLLS program developed by Freed and coworkers [17,18]. This program, which allows a single spectrum to be fitted with two components having different mobilities and magnetic tensor parameters, gives the relative populations and the associated rotational correlation times. Similar to previous studies [12,19,20], these parameters were determined based on a general analysis of the overall spectra obtained from this work and once determined, all the EPR spectra were simulated using the same previously established values.

3. Results

3.1. EPR spectra of spin-labeled SC samples

The experimental and best-fit EPR spectra at 24 °C for three doxyl spin-labeled lipids (Fig. 1) structured in the SC membranes are shown in Fig. 2 at the indicated ethanol concentrations. An examination of these EPR spectra reveals that they are composed of one to three spectral components, each indicating a different motional state of spin probes. Spectral simulations allowed to separate and obtain the contribution of each component in the composed EPR spectra as well as to evaluate the mobility of each one. The third component (see arrows in Fig. 2) corresponds to the spin label fractions that are in the SC tissue but outside of the membranes and freely tumbling in the solvent. In fact, the line shape of this component is very similar to the EPR spectrum of spin labels free in the buffer (bottom middle column). Concerning the most hydrophobic spin probes used here, 5-DMS, the free signal arises only at higher ethanol concentration (70%), whereas for 5-DSA it appears at 50%. For the samples with the androstanol analog (ASL) and containing 50–70% ethanol, the free form represents a substantial fraction in the composed EPR spectra.

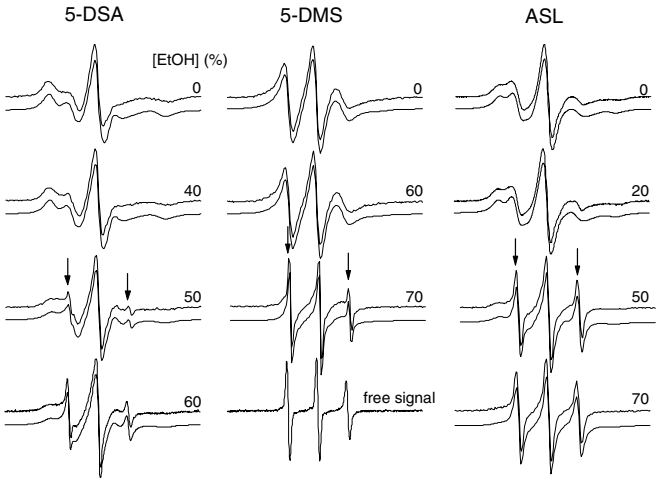

Fig. 2. Experimental (lines) and best-fit (lines below the experimental ones) EPR spectra of spin labels stearic acid (5-DSA) and the methyl stearate (5-DMS) derivatives and the steroid ASL in stratum corneum with the presence of increasing ethanol concentrations, indicated for each spectrum in the figure. The best-fit spectra in this study were obtained by NLLS fitting, using a simulation model with two or three spectral components. The arrows indicate the features of the spectra where arises the free signal. The total scan range of the magnetic field was 100 G.

The experimental EPR spectrum of ASL spin label in SC membranes with 20% ethanol in the buffer is shown in Fig. 3a. The simulated EPR spectrum and its respective spectral components isolated by NLLS program are also presented. The EPR spectra of ASL and 5-DSA spin labels in SC membranes are shown for several L-menthol concentrations in Figs. 3b and c, respectively. The free component consistent with spin probes extracted from SC membranes is not present over the whole ethanol concentration range. It can be noticed from the spectra that the relative population of the component 1 decreases gradually by increasing the menthol concentration, tending to disappear at about 1% L-menthol.

Components 1 and 2 represent a bimodal distribution of spin labels in the membrane. The origin of these two components has been discussed in a previous paper [12] and is not yet finally established. The more plausible interpretation is that component 1 arises from a population of spin labels that are hydrogen-bonded to the polar surfaces of the membranes, while the less motionally restricted population (component 2) is generated by nonhydrogen-bonded spin labels and more deeply inserted in the hydrophobic core.

3.2. Lipid chain dynamics and partition of spin labels in SC

The rotational motion parameters $R_{bar}1$ and $R_{bar}2$, obtained from the fittings of EPR spectra, reflect the mobility states of components 1 and 2, respectively. In Fig. 4, these parameters, converted to nanosecond scale, are plotted as a function of the ethanol concentration in the SC samples (Fig. 4a) and L-menthol concentration added to the solvent with 20% ethanol (Fig. 4b). As illustrated in Fig. 4a, the 5-DMS was considerably more mobile as compared to the 5-DSA and ASL. However, no significant mobility changes were observed for the three spin labels as a function of ethanol concentration, indicating that ethanol did not alter the membrane fluidity. On the other hand, the addition of L-menthol caused increases in the mobility of component 2. The effect with 1% L-menthol is already close to that observed at higher terpene concentration.

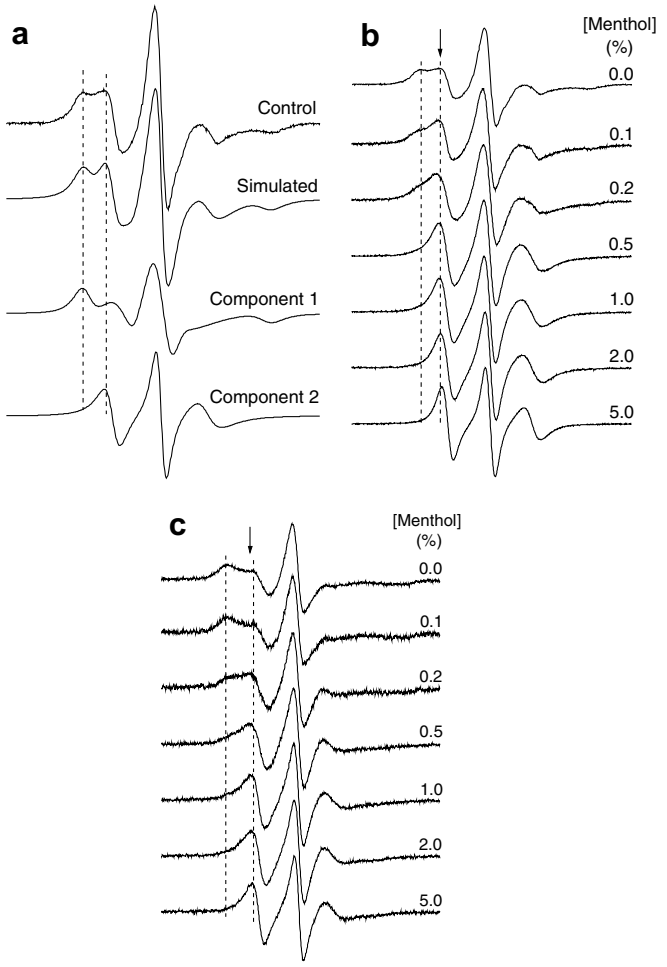

Fig. 3. (a) Experimental EPR spectrum of spin label ASL in stratum corneum (control) and its simulated spectrum obtained by NLLS fitting using a model with two spectral components (components 1 and 2). The dashed lines indicate the magnetic field positions where the features of the spectral components 1 and 2 are more clearly visible (see the text for details). (b and c) EPR spectra of spin labels 5-DSA and ASL, respectively, in stratum corneum with 20% ethanol in the buffer (pH 5.1) and the indicated L-menthol concentrations. The total scan range of the magnetic field was 100 G.

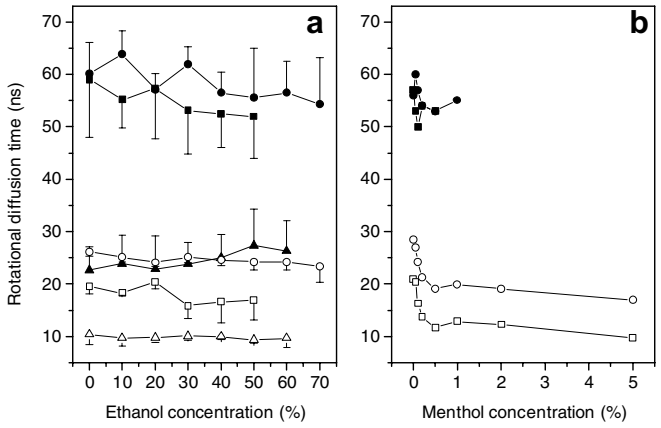

Fig. 4. Rotational diffusion coefficient, R_{bar}, for spin-labeled lipids in stratum corneum as a function of ethanol (a) and L-menthol (b) concentrations. The concentration of L-menthol was varied in presence of 20% ethanol in the solvent. The parameters were obtained from the simulation of EPR spectra, considering the resolution of two spectral components of spin labels in the membrane. Symbols: 5-DSA (circles), ASL (squares) and 5-DMS (triangles); closed and open symbols refer to R_{bar} of components 1 and 2, respectively. In (a) the means and SD are from three-independent experiments.

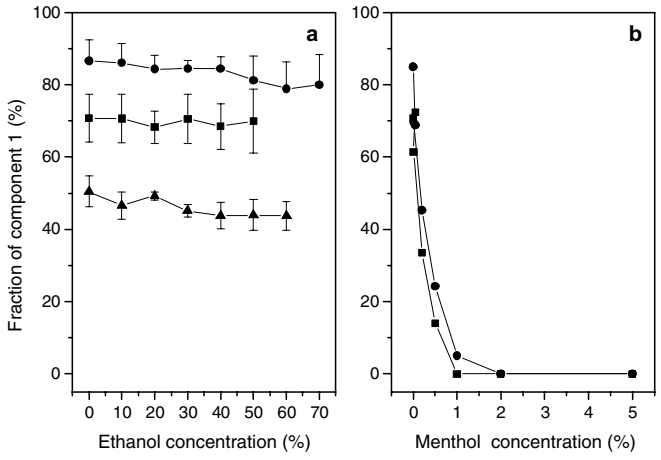

Fig. 5. Fraction of the more restricted component 1 in the EPR spectra of spin-labeled lipids in stratum corneum as a function of ethanol (a) and L-menthol (b) concentrations. L-menthol was added to solvent with 20% ethanol. Symbols: 5-DSA (circles), ASL (squares) and 5-DMS (triangles). Values in (a) are means ± SD ($n = 3$).

Figs. 5a and b represent the fraction of spin labels associated to the component 1 plotted as a function of ethanol and L-menthol concentrations, respectively. It is noted that the spin-probe population forming the components 1 did not change significantly in the presence of ethanol for the three spin labels, suggesting that the formation of hydrogen bonds between these probes and the polar surfaces of the membranes is essentially preserved with the gradual addition of ethanol up to 70%. In fact, the line shapes of these spin labels remained basically the same over the whole ethanol concentration range. In contrast, the Fig. 5b shows clearly that in presence of 1% L-menthol, the component 1 of both ASL and 5-DSA tends to disappear.

The fraction of extracted spin probe increased dramatically for ASL at ethanol concentrations above 30%, as shown in Fig. 6. These findings also reveal the minor ability of ethanol to extract the spin probe 5-DMS, whereas an intermediary level of extraction was observed for 5-DSA.

3.3. Solvent content in SC samples

As the amount of extracted spin labels from membranes should be proportional to the solvent content in the samples, they were carefully prepared so as to maintain the same solvent volume on the SC tissue. Gravimetric measurements of the solvent content in the samples showed gradual reductions with increasing ethanol concentrations (Table 1), indicating better packing of the SC tissue in the samples containing ethanol in the solvent.

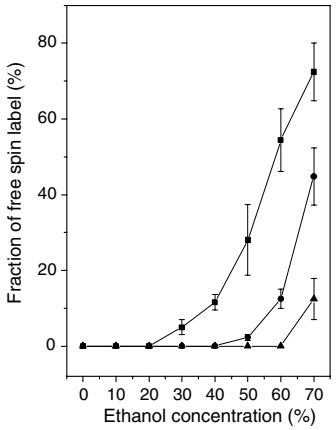

Fig. 6. Fraction of spin-labeled lipids extracted from stratum corneum membranes as a function of ethanol concentration. Symbols: 5-DSA (circles), ASL (squares) and 5-DMS (triangles). The means and SD are from three-independent experiments.

Table 1
Solvent content in the SC samples

Ethanol:buffer (%, v/v)	Solvent:SC (%, w/w)[a]	Solvent:SC (%, v/w)
0	72.6 ± 3.6[b]	72.6 ± 3.6
20	67.8 ± 1.5	70.7 ± 1.5
40	61.4 ± 2.0	67.0 ± 2.2
60	57.5 ± 2.8	65.1 ± 3.1

[a] Ratio between weight of solvent to weight of wet SC in percentage.
[b] Values are means ± SD ($n = 3$).

4. Discussion

The binding of ethanol molecules to the lipid matrix of biological membranes is an issue not well understood yet, but of great interest due to the role of ethanol in metabolism, drug delivery, toxicity and local tolerance. Recently, through a combination of nuclear magnetic resonance measurements and molecular dynamic simulations it has been found that ethanol interacts with phospholipid bilayers primarily via hydrophilic interactions such as hydrogen bonds and that it can penetrate the region of upper chain segments [21]. However, the probability of locating ethanol in the center of the bilayer is three orders of magnitude lower than locating ethanol at the lipid/water interface [21]. Another recent study supports the view that alcohols partitioning into the bilayer headgroup region, with enhanced partitioning as the chain-length of the alcohol increases, result in the reduction of interfacial tension and increase of membrane permeability [22].

Fourier transform infrared (FTIR) measurements of human SC did not detect significant changes in the lipid alkyl chain packing, mobility and conformational order in the presence of the short chain alcohols. Furthermore, a decrease of the alkyl chain absorbance suggests lipid extraction [23]. Similarly, using FTIR technique, a reduction in the mobility of SC lipid alkyl chains in the presence of ethanol has also been observed [24]. Fluorescence anisotropy studies on the interaction of the short chain *n*-alkanols with liposomes composed of SC lipids showed small or no increase in fluidity in the deep hydrophobic region close to the center of the bilayer [25]. On the other hand, slight increases in fluidity close to the hydrophobic/hydrophillic interface (C2–C9) were recorded. In the present work, alterations of membrane polarity caused by ethanol were also not detected, which is in agreement with the aforementioned studies by fluorescence [25].

These findings indicate that a number of compensatory effects of ethanol in membranes are present. Ethanol interacts with the lipid matrix primarily via hydrogen bonds and the probability of its penetration into the membrane hydrophobic core is extremely low when in a molar ratio of lipid/ethanol/water of 1:1:10 [22]. Its location at the polar interface of the membranes should disturb the natural microstructure of the lipids, increasing the area per headgroup and, consequently, reducing the superficial tension [22]. Therefore, it would be expected to cause an increase in fluidity. Beyond that, a possible cholesterol extraction would also contribute by increasing the membrane dynamics since cholesterol reduces membrane fluidity [26,27]. Nevertheless, ethanol should have other compensatory effects such as dehydration in the polar groups caused by local displacement of water molecules, favoring the formation of direct hydrogen bonds between the lipids.

Ethanol has been currently used in commercial transdermal delivery systems as a potent cosolvent of low systemic toxicity and local tolerability. Furthermore, it is well known that ethanol is also a moderate skin permeation enhancer and has a synergism with other permeation accelerants such as the terpenes. In this study we perform careful measurements exploiting the high sensitivity of EPR spectroscopy with spin-labeled nitroxides in order to explore if ethanol could alter the lipid fluidity and relative fractions of the spectral components 1 and 2 of the SC membranes. According to our previous interpretation on the origin of these two components [12], mentioned in the results section, they could be involved in the mechanisms of molecule permeation across membranes. Component 1 is assigned to a fraction of spin labels that are hydrogen-bonded to the polar headgroups of the membranes, (rigid structure) while component 2 is generated by those spin labels that are hydrogen-bonded to water molecules (mobile structure) or momentarily nonhydrogen-bonded and more deeply inserted in the hydrophobic core. Similarly, it has been previously described a bimodal distribution of the fluorescent probe 4′-dimethylamino-3-hydroxyflavone (probe F) in phospholipid bilayer [28], where two probe locations characterized by different polarities and hydrations were identified and attributed to the ability of the probe to participate in intermolecular H-bonding. Nonetheless, our findings indicate that ethanol did not alter either the dynamics of SC membranes of neonatal rats, or the relative fractions between the two spectral components, as deduced from EPR parameters of four lipid spin labels (data not shown for 16-DSA). The results suggest that ethanol acts as a selective lipid extractor of SC

membranes and that for higher ethanol concentration the amount of extracted lipid can be very high.

On the other hand, by increasing the L-menthol concentration in the solvent containing 20% ethanol, a gradual increase in the fluidity of SC membranes is observed together with a gradual interchange of spin labels from component 1–2. The fitting program NLLS allowed a detailed analysis of the EPR spectra. In the absence of L-menthol, the rotational diffusion time is essentially due to contribution of spin labels forming the component 1, which are maintained at 50–60 ns for 5-DSA and ASL. With the addition of terpene, the spin probes are transferred from this more restricted component 1 to the more mobile component 2, with rotational diffusion time of ~20 ns for 5-DSA and ~13 ns for ASL at 1% L-menthol and changing, respectively, to ~17 and 10 ns at 5% L-menthol. Essentially, the event that occurs in the presence of L-menthol is the passage of spin-labeled lipids from a more to a less restricted spectral component. Probably, an appreciable fraction of L-menthol stabilizes in the central region of the membranes increasing the mobility and the lipid volume in this region. This process introduces the polar group of the L-menthol molecule inside the membrane and attracts the membrane lipids to the center of membrane (greater fraction of component 2), becoming the membrane interior an environment still more polar. The rupture of hydrogen bonding network in the polar interface associated to the transitory displacements of polar groups towards the hydrophobic core would lead to a loss of integrity in the SC barrier, facilitating the partitioning and consequently the permeation of polar molecules such as AZT.

The EPR results from this work are consistent with ATR-FTIR studies using a simple model SC lipid, where the presence of 1,8-cineole and L-menthol reduced the non-hydrogen-bonded amide I stretching frequency [8]. The authors have concluded that terpenes mainly act at polar headgroups and break the inter- and intralamellar hydrogen bonding network. We believe that these EPR data provide new insights into the mechanisms of molecule permeation in SC membranes facilitated by ethanol and L-menthol. However, for a better understanding, additional experiments will be carried out using simple models of membranes with a smaller complexity as compared to the one of SC.

Acknowledgements

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