Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 350 (2008) 103–112
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

# Terpenes increase the partitioning and molecular dynamics of an amphipathic spin label in stratum corneum membranes

Jorge Luiz Vieira dos Anjos, Antonio Alonso *

*Instituto de Física, Universidade Federal de Goiás, Goiânia 74001-970, GO, Brazil*

Received 24 July 2007; received in revised form 20 August 2007; accepted 20 August 2007
Available online 24 August 2007

**Abstract**

In this work, the interaction of the skin penetration enhancers DL-menthol, α-terpineol, 1,8-cineole and (+)-limonene with the uppermost skin layer, the stratum corneum and with multilamellar vesicles from 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) is investigated by electron paramagnetic resonance (EPR) spectroscopy of the small spin label 2,2,6,6,-tetramethylpiperedine-1-oxyl (TEMPO), which partitions the aqueous and hydrocarbon phases. The EPR spectrum allows for the determination of the actual partition coefficient and the rotational diffusion rates of the spin probe in the two environments. The enthalpy changes, $\Delta H^{\circ}$, to transfer the spin probe from the aqueous to the hydrocarbon phase, as well as the activation energies associated to its rotational motion, were considerably smaller for stratum corneum, indicating less pronounced thermal reorganization. For DPPC, the terpenes increased both the partition coefficient and the rotational diffusion rate of the spin label in the membrane, except in the liquid–crystalline phase, while these increases in stratum corneum were observed in the entire temperature range measured with the exception of the rotational motion parameter for DL-menthol and α-terpineol at temperatures below their melting point (32–41 °C). It is suggested that the terpenes effectively acting as spacers in the membrane fluidize the lipids and cause ruptures in the hydrogen-bonded network of the polar interface.


*Keywords:* Stratum corneum; EPR; Spin label; Lipid dynamics; Terpene

## 1. Introduction

The effectiveness of transdermal drug delivery depends on the drug's ability to penetrate the skin sufficiently to reach therapeutic levels. The main skin barrier to exogenous chemical absorption is the outer epidermal layer, the stratum corneum (SC), composed of corneocytes surrounded by a multilamellar lipid matrix. Corneocytes are keratin-filled dead cells containing an insoluble cell envelope of cross-linked proteins, which reduce absorption of drugs into the cells (Bouwstra and Honeywell-Nguyen, 2002), and a cell lipid envelope composed mainly of ω-hydroxyceramides covalently bound to the periphery of the cell envelope (Wertz and Downing, 1987). The intercellular region contains a complex lipid mixture, ordered in multilayered structures known as lipid lamellae and consisting mainly of ceramides, free fatty acids, cholesterol and cholesteryl sulfate.

The most widely implemented approach to overcoming this skin barrier has been the use of chemical penetration enhancers, which ideally alter the physicochemical nature of the SC safely and reversibly to facilitate the drug's delivery through the skin. According to the lipid–protein-partitioning theory (Barry, 1991), penetration enhancers may increase the permeability of a drug by affecting the intercellular lipids of the SC via extraction or fluidization (Yamane et al., 1995), and/or by increasing the partitioning of the drug in the SC membranes (Gao and Singh, 1998), and/or by changing conformations within the keratinized protein component (Williams and Barry, 2004).

Terpenes are naturally occurring compounds derived from essential oils, which contain only carbon, hydrogen and oxygen atoms. Generally used in flavorings, perfumes and medicines, many terpenes including, 1,8-cineole, menthol and α-terpineol, are claimed to be generally recognized as safe (GRAS) materials. Their interactions with the SC are of interest to understand how

*Abbreviations:* SC, stratum corneum; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; TEMPO, 2,2,6,6-tetramethyl-piperidine-1-oxyl; EPR, electron paramagnetic resonance; NLLS, nonlinear least-squares fitting program.

* Corresponding author. Fax: +55 62 3521 1014.
*E-mail address:* alonso@fis.ufg.br (A. Alonso).

0378-5173/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2007.08.024

terpenes, and other small amphiphilic molecules, enhance the permeability of skin. Recently, it was reported that 1,8-cineole and L-menthol applied at 5% (w/v) in solution with 66% ethanol (v/v) provide sufficient zidovudine (AZT) flux in rat skin (more permeable than human skin) to reach its therapeutic concentration (Narishetty and Panchagnula, 2004, 2005). Terpenes have been reported as permeation enhancers of several polar and non-polar drugs such as 5-fluorouracil (Cornwell and Barry, 1994), morphine hydrochloride (Morimoto et al., 2002), propranolol hydrochloride (Hori et al., 1991), imipramine hydrochloride (Jain et al., 2002), indomethacin (Okabe et al., 1989), hydrocortisone (El-Kattan et al., 2000), tamoxifen (Gao and Singh, 1998) and haloperidol (Vaddi et al., 2002a). However, there are only few studies on mechanisms of permeation enhancement by terpenes in SC, which use mainly Fourier-transformed infrared spectroscopy (FTIR), differential scanning calorimetry (DSC) (Vaddi et al., 2002b; Narishetty and Panchagnula, 2004, 2005) and small-angle X-ray diffraction (SAXD) (Cornwell et al., 1996). ATR-FTIR experiments showed that both 1,8-cineole and L-menthol increase $CH_2$ stretching frequencies and decrease the mid transition temperature ($T_m$) of a model SC lipid system (Narishetty and Panchagnula, 2005). SAXD experiments showed that D-limonene and 1,8-cineole in propylene glycol decrease the intensity of lipid based reflections, suggesting disruption of lipid packing within the bilayers, while DSC thermograms of these same systems indicated that the terpenes reduce the temperatures of the two major lipid transitions of human SC at 72 and 83 °C (Cornwell et al., 1996).

Spin-labeling electron paramagnetic resonance (EPR) has been employed to obtain information about SC membranes in the intact tissue (Alonso et al., 1995, 1996, 2000; Queirós et al., 2005). It has been shown that hydration of SC increases lipid fluidity (Alonso et al., 1995, 1996), and that the lipid dispersions prepared with extracted SC lipids are much more fluid than the lipids in the intact tissue, whereas the ceramides from the corneocyte lipid envelope, which are covalently bound to the SC proteins, have the lowest state of mobility among the SC lipids (Alonso et al., 2000). The EPR spectra of lipid spin labels in SC membranes are characterized by the coexistence of two spectral components with very different states of mobility. In previous work (Queirós et al., 2005), the origin of these two spectral components was interpreted based on the ability of spin labels to participate in intermolecular hydrogen bonding into the membrane. The more motionally restricted component was assigned to a class of spin probe hydrogen bonded to the polar headgroups (more rigid structure) and the more mobilized component was attributed to those spin labels temporarily nonhydrogen bonded to the polar interface and more deeply inserted in the hydrophobic core. Recently, the effect of the terpenes L-menthol and 1,8-cineole on the SC lipid dynamics was examined in detail (Anjos et al., 2007, 2007a). The presence of 1% terpenes in the solvent drastically increased the lipid fluidity, especially by transferring the spin probes from a more to a less motionally restricted spectral component into the membranes. Furthermore, these two terpenes increased the rotational diffusion rates only of spin probes from the more mobilized component.

The small amphipathic spin label TEMPO partitions between the bilayer and aqueous phase of many membranes (Smirnov et al., 1995). The EPR spectra generally observed for this spin probe in membranes are composed of two spectral components differing in mobility and polarity. The NLLS fitting program allows for the separation of these components, determining their relative fractions and rotational diffusion rates. Since penetration enhancers generally act by favoring drug partition in SC membranes and the spin label TEMPO may mimic drugs, in the present work, we examined the capacity of four terpenes to increase the partitioning and mobility of this spin probe in the SC lipid domain. This small spin probe provides novel findings on the effects of terpenes in SC membranes. Unlike the other spin labels used in previous works (Anjos et al., 2007, 2007a), which are essentially stabilized in the membrane, this spin label allows its partition coefficient between the aqueous and hydrocarbon phases to be monitored. Furthermore, being a small molecule with larger diffusion capacity through the membrane, its rotational motion more accurately reflects the global dynamics of the membrane and its phase transitions. Because the lipid composition of SC is complex, we conducted a parallel study with DPPC membranes to compare the results for SC with those of a simpler and well-known model.

## 2. Materials and methods

### 2.1. Preparation of SC membranes

SC membranes of neonatal Wistar rats less than 24 h old were prepared as described previously (Anjos et al., 2007, 2007a). After the animal was killed, its skin was excised and fat was 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 internal side in contact with the water for 2 h. The external side was placed in contact with a filter paper and the SC sheet was carefully separated from the remaining epidermis. Subsequently, the SC was transferred to a Teflon-coated screen, washed with distilled water and allowed to dry at room temperature. The membranes were stored with 1 L of silica gel in a desiccator under a moderate vacuum.

### 2.2. Spin labeling and treatment of SC

To prevent nitroxide reductions, the sulfhydryl groups of the SC tissue were blocked by incubating the SC membranes in a solution of 50 mM *N*-ethyl maleimide (Sigma Chem. Co., St. Louis, MO, USA) for about 15 h. An intact piece of SC (3 mg) was then rinsed with 1.5 mL Milli-Q water and incubated for 90 min at room temperature in 45 μL of acetate-buffered saline (10 mM acetate, 150 mM NaCl and 1 mM EDTA, pH 5.5) containing 0.2 mM spin label TEMPO (Fig. 1) purchased from Sigma Chem. Co., and the corresponding terpene concentration (Acros Organics, Geel, Belgium), which was added to the sample in 5 μL of ethanol. The control samples were subjected in an identical experiment, in which 5 μL of ethanol was added without the terpenes. The intact SC membrane was then intro-