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In vitro studies on penetration of terpenes from matrix-type transdermal systems through human skin

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Abstract

Polyurethane matrices containing up to 39% of the terpenes eucalyptol, L-limonene, D-limonene, dipentene or terpinolene were produced. Release of the terpenes directly to the acceptor fluid, as well as through isolated human epidermis and dermis, was studied. In the presence of dermis the penetration profiles were very similar to the release profiles, indicating that dermis does not present a barrier for penetration of terpenes. For all terpenes the penetration was slower in the presence of epidermis (K_p was in the range $0.21–1.8 \times 10^{-3}$ cm/h). Release and penetration through the epidermis and dermis were fastest for dipenetene (mixture of D-limonene and L-limonene), being at least 3–4 times faster than for D-limonene and L-limonene. Large amounts of terpenes found in epidermis (approximately 1.5 mg/cm^2) indicate that affinity of these compounds to the stratum corneum is very high.

Keywords: Skin penetration; Terpenes; Eucalyptol; Limonene; Terpinolene; Enantiomers; Transdermal system

1. Introduction

Penetration enhancers are substances which rapidly and reversibly promote percutaneous penetration of drugs. Some of the most frequently used compounds are alcohols, fatty acids and terpenes (Williams and Barry, 1992). Penetration enhancers, with significant biological activity and the possibility of causing side effects, should not or only in restricted quantities penetrate through the skin. Therefore, not only the promotion of penetration of other drugs but also their own penetration should be better examined.

We previously reported the promoting effects of glyceryl trioleate, oleic acid and propylene glycol on percutaneous absorption of isosorbide dinitrate. Their absorption into and through human skin in vitro was documented, and plasma concentrations of oleic acid or propylene glycol in rats were found to be comparable with concentrations reported for some drugs (Gabiga et al., 1996, 1997, 2000; Cal et al., 1997).

Terpenes are hydrocarbons with the general formula $(C_5H_8)_n$ and among them are also oxygenic derivatives. Plant essential oils are the main source of terpenes. Mono-, sesqui- and diterpenes, alone or in mixtures, are employed as percutaneous penetration enhancers. They are most often

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explored to increase penetration of non-steroidal anti-inflammatory drugs.

The enhancement of skin penetration of drugs is explained as a result of increased drug solubility in the stratum corneum treated with terpenes. Terpenes interact with the intercellular lipids, perturbing their lamellar packing. For lipophilic drugs terpenes increase the partition coefficient stratum corneum/vehicle. The permeation of lipophilic drugs increases proportionally to their solubility in the enhancer (or enhancer's solution) while penetration of hydrophilic drugs is improved due to increased diffusion coefficient (Williams and Barry, 1991, 1992; Kobayashi et al., 1993; Cornwell et al., 1996; Moghimi et al., 1996, 1997; Gao and Singh, 1997).

Table 1
Composition of TTS matrices

	Terpene		Polyurethane
	mg/cm^2	% (w/w)	mg/cm^2
D-Limonene	5.36	12.7	36.54
L-Limonene	5.92	13.9	36.54
Dipentene [a]	9.15	26.6	25.31
Terpinolene	3.96	9.8	36.54
Eucalyptol	15.98	38.7	25.31

[a] Content of limonene enantiomers in dipentene not determined.

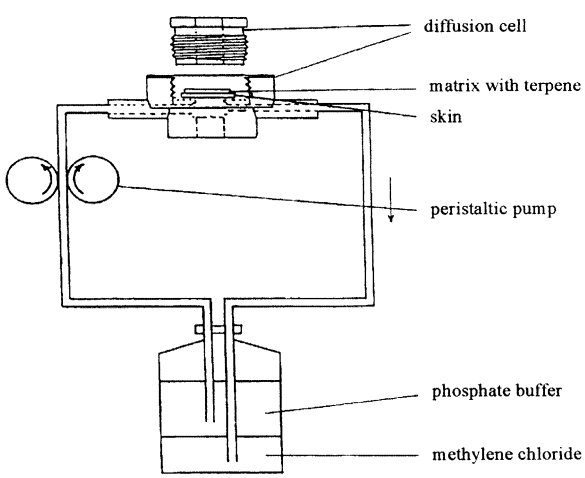

Fig. 1. The schematic diagram of instrumentation for penetration studies of terpenes.

Since some terpenes increase penetration of ionized drugs, it is suggested that they may also influence the polar intercellular pathway of penetration (Katayama et al., 1992; Sznitowska et al., 1998; Obata et al., 1999).

Terpenes are not only used as penetration enhancers, but even more often are present in drugs and cosmetics as components of essential oils added for some other reasons: for inhalation or for topical administration, as rubefacient, analgesics or antiseptics.

There are very limited published studies concerning the penetration of terpenes through the skin (Sugibayashi et al., 1995; Obata et al., 1999; Janicki et al., 2000) or mucosal membrane (Ceschel et al., 2000). These studies mainly refer to liquid or semisolid forms, however there are no available data on more specialized formulations, i.e. transdermal therapeutic systems (TTS). Therefore, the aim of this investigation was to evaluate in vitro percutaneous penetration of the selected terpenes released from model matrices. Five compounds, namely D-limonene, L-limonene, dipentene, terpinolene and eucalyptol, were chosen for the study. In order to avoid any interference the matrices contained single terpenes and no drug was present.

2. Materials and methods

2.1. Materials

Eucalyptol (purity ~99%), D-limonene (~97%), L-limonene (~97%), terpinolene (~90%) and L-carvone (~99%) used as an internal standard (IS) in analytical procedures were purchased from Fluka (Switzerland). Dipentene (~97%) was a gift from Pollena-Aroma (Poland). The terpenes were stored at room temperature. Methanol (P.O.Ch., Poland) was HPLC grade. Polyurethane PU 1/89 (Celwiskoza, Poland) was used as a matrix polymer.

2.2. Preparation of matrices

Polyurethane was dissolved in dimethylformamide in the ratio 1:20 (w/w). TTS matrices

Table 2
The parameters describing kinetics of the release of terpenes directly into acceptor fluid (A) or penetration through dermis (D) and epidermis (E). Each value represents the mean ± SD, $n = 4$

	A	D	E	Statistical analysis [b]
D-Limonene				
Q_8 (mg/cm^2) [a]	1.52 ± 0.08	1.23 ± 0.06	0.41 ± 0.04	A > D > E
(%)	28.36	22.95	7.65	
J_{ss} (mg/cm^2/h)	0.24 ± 0.02	0.20 ± 0.02	0.08 ± 0.01	A = D > E
K_p (cm/h) $\times 10^{-3}$	1.68 ± 0.16	1.44 ± 0.16	0.55 ± 0.04	A = D > E
T_{lag} (h)	2.18 ± 0.22	2.46 ± 0.18	1.78 ± 0.03	A = D > E
L-Limonene				
Q_8 (mg/cm^2)	2.03 ± 0.11	1.56 ± 0.14	0.14 ± 0.02	A > D > E
(%)	34.29	26.35	2.36	
J_{ss} (mg/cm^2/h)	0.27 ± 0.01	0.21 ± 0.03	0.03 ± 0.01	A > D > E
K_p (cm/h) $\times 10^{-3}$	1.75 ± 0.16	1.38 ± 0.17	0.21 ± 0.06	A > D > E
T_{lag} (h)	1.19 ± 0.27	1.33 ± 0.24	1.77 ± 0.17	A = D < E
Dipentene				
Q_8 (mg/cm^2)	3.78 ± 0.23	3.32 ± 0.26	2.08 ± 0.30	A > D > E
(%)	41.43	36.28	22.73	
J_{ss} (mg/cm^2/h)	0.50 ± 0.05	0.50 ± 0.03	0.38 ± 0.06	A = D > E
K_p (cm/h) $\times 10^{-3}$	2.37 ± 0.22	2.37 ± 0.15	1.80 ± 0.27	A = D > E
T_{lag} (h)	1.26 ± 0.33	1.65 ± 0.09	2.60 ± 0.11	A = D < E
Terpinolene				
Q_8 (mg/cm^2)	1.12 ± 0.12	0.97 ± 0.12	0.46 ± 0.12	A = D > E
(%)	28.28	24.49	11.62	
J_{ss} (mg/cm^2/h)	0.19 ± 0.02	0.17 ± 0.02	0.08 ± 0.02	A = D > E
K_p (cm/h) $\times 10^{-3}$	1.65 ± 0.21	1.52 ± 0.18	0.71 ± 0.16	A = D > E
T_{lag} (h)	2.45 ± 0.22	2.80 ± 0.12	1.798 ± 0.07	D > A > E
Eucalyptol				
Q_8 (mg/cm^2)	3.42 ± 0.26	3.14 ± 0.19	2.06 ± 0.08	A = D > E
(%)	21.40	19.65	12.89	
J_{ss} (mg/cm^2/h)	0.44 ± 0.03	0.46 ± 0.06	0.37 ± 0.04	A = D > E
K_p (cm/h) $\times 10^{-3}$	1.36 ± 0.09	1.41 ± 0.18	1.14 ± 0.12	A = D > E
T_{lag} (h)	1.14 ± 0.10	1.22 ± 0.08	2.50 ± 0.23	A = D < E

[a] Q_8 cumulative amount of terpenes found in the acceptor fluid after 8 h, expressed in mass per unit area and as a per cent of the total dose.
[b] = no significant difference; < or > significant difference.

were obtained by casting the solution on siliconized glass plates (diameter 7.0 cm), followed by evaporation of the solvent in an air-conditioned chamber for 72 h in relative humidity 50% and temperature 50 ± 1 °C. In preliminary studies it was discovered that more than 50% of the terpenes evaporated when dissolved in dimethylformamide together with polymer. Hence, the loading was performed by immersing the blank matrices, cut into circles 1.6 cm in diameter, in an investigated pure terpene for 24 h. The excess of terpene was removed with blotting paper. The final contents of terpenes in matrices are shown in Table 1.

2.3. Release and skin penetration studies

The release and penetration studies were performed in flow-through Teflon diffusion cells (Crown Glass Co., USA) with diffusion area of 0.65 cm^2. The cells were kept in a thermostated block at 37 °C. The matrices were placed on the skin mounted in the cells. In order to prevent evaporation of terpenes, the surface of matrices

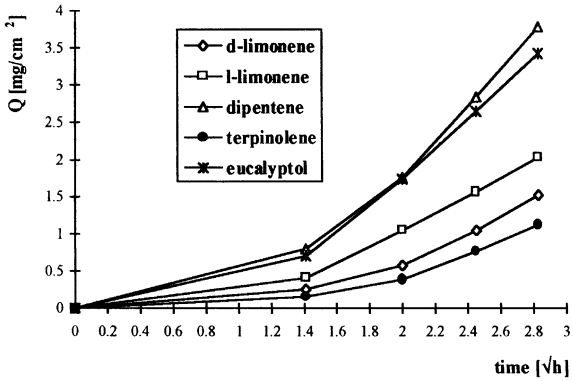

Fig. 2. The release of terpenes from TTS matrices directly into the receiver fluid ($n = 4$).

was protected by aluminium foil laminated with polyester (3M Medica, USA). Isotonic phosphate buffer pH 7.3 (5.1 g $NaH_2PO_4 \cdot 2H_2O$ and 14.8 g Na_2HPO_4 in 1000 ml of water), 10 ml, was recirculated under the skin with a flow rate 10 ml/h. The release of the terpenes was determined under the same conditions, without the skin as a barrier.

Considering the limited solubility of terpenes in the buffer (< 1 mg/ml), a two-phase acceptor system was employed. It was obtained by addition of 5 ml of methylene chloride to the tube serving as a reservoir of the buffer (Fig. 1). An immediate partitioning of terpenes from the acceptor buffer to methylene chloride occurred, while the matrices or the skin were only in contact with the phosphate buffer.

Human cadaver skin was obtained from a thorax with permission of the Local Independent Committee For Clinical Trials. The skin was stored frozen at -20 °C prior to the experiments. The skin layers (epidermis and dermis) were isolated by the heat separation technique (Kligman and Christophers, 1963). The whole skin was immersed in water at 60 °C for 45 s, followed by careful removal of the epidermis. The obtained skin layers were dried with blotting paper and placed in the diffusion cells. The system was equilibrated for 1 h with the recirculating buffer. The samples of methylene chloride (0.5 ml) were collected after 2, 4, 6 and 8 from the bottom layer in the reservoir tube and replaced by a fresh solvent. When the experiment was terminated, the skin was removed and extracted as described below.

2.4. Determination of terpenes in matrices, acceptor fluid and skin

The matrices were extracted twice with 5.0 ml methanol containing IS (1 mg/ml). Dermis and epidermis were extracted twice with 1.0 ml methanol containing IS (5 mg/ml). The extraction was performed in glass vessels with Teflon caps (Chromacol, USA). The samples were centrifuged at $1920 \times g$ for 15 min. Methanol phase was filtered through a 0.22 μm membrane filter (Sartorius, Germany) and analysed. Fractions of the

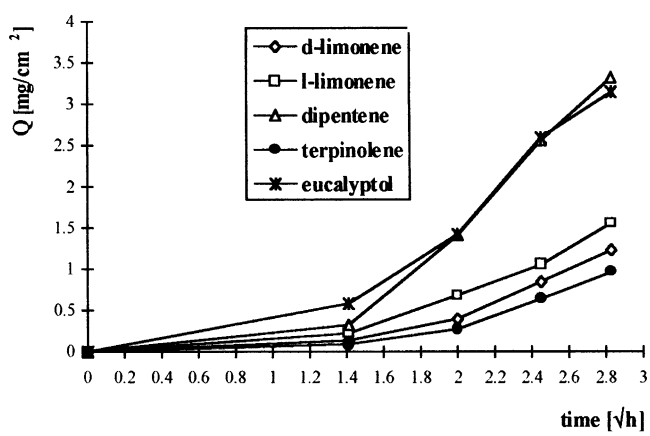

Fig. 3. Dermal penetration of terpenes from TTS matrices into the receiver fluid ($n = 4$).

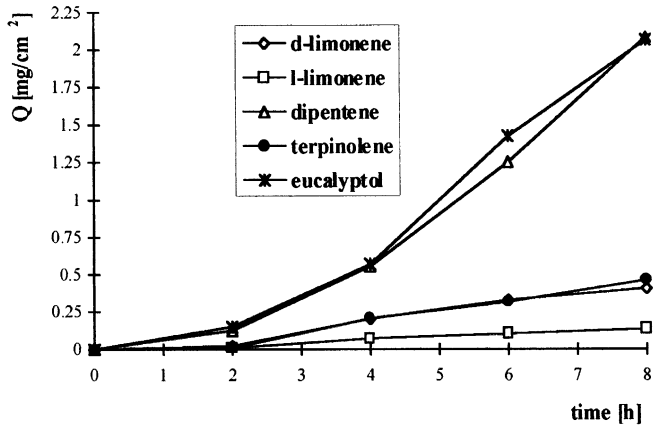

Fig. 4. Epidermal penetration of terpenes from TTS matrices into the receiver fluid ($n = 4$).

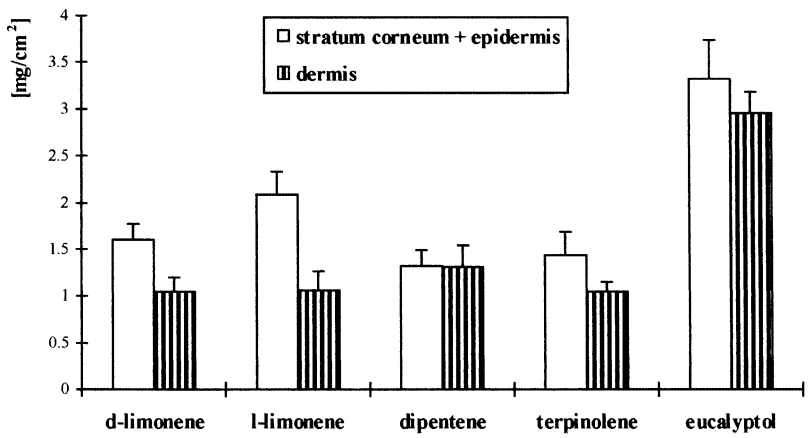

Fig. 5. Cumulation of terpenes in epidermis and dermis after penetration studies ($n = 4$).

methylene chloride 0.5 ml in volume were mixed with 0.5 ml methanol containing IS (50 μg/ml) and filtered through a membrane filter. Two microliter aliquots were injected in triplicate onto a gas chromatograph. The system consisted of a Carlo Erba HRGC 5300 Mega series apparatus equipped with a flame ionization detector, a 'fused silica' capillary column 30 m × 0.53 mm i.d. with a 1.0 μm stationary phase SPB-5 [poly(5%-diphenyl–95%-dimethylsiloxane)] (Supelco, USA) with a 5 m deactivated precolumn. Chromatographic conditions were as follows: carrier-gas (hydrogen) flow rate was 6.5 ml/min, injection was done by a 'cold-on-column' method, the oven temperature was 80 °C, the detector temperature was 250 °C. The areas of peaks were measured by a one-channel integrator Data Jet (Spectra Physics Analytical, USA).

2.5. Data analysis

The cumulative amounts of terpenes penetrating per unit skin area (Q) were plotted against time or square root of time. From the slope of the linear portion of the plot a steady-state flux (J_{ss}) was determined. The permeability coefficient (K_p) was calculated as the ratio of J_{ss} and the initial concentration of terpene in the matrix. T_{lag} was estimated by extrapolation of the linear portion of the plot to the time axis.

The results of release and penetration experiments were expressed as the mean ± SD. Statistical differences were established by one-way ANOVA. Differences were considered significant at $P < 0.05$.

3. Results and discussion

Percutaneous penetration of five different terpenes from the matrix-type transdermal patches through the isolated skin layers and directly to an acceptor media was investigated.

Terpenes were incorporated in the polyurethane matrix by sorption, and different amounts of terpenes accumulated by the matrices (Table 1) demonstrate the affinity of terpenes to the polymer. The highest loading (27–39%) was achieved for eucalyptol and dipentene, while for other terpenes the absorbed amounts were 2–3 times lower. Macroscopic and microscopic observations revealed that all terpenes existed also in a dispersed phase.

The release rate from the matrix directly to the acceptor media was the largest for terpenes for which concentrations in the matrices were the highest, i.e. for eucalyptol and dipentene (Table 2, Fig. 2). When permeation coefficients are compared, the values increase in the following order: L-limonene < D-limonene < terpinolene < eucalyptol < dipenetene. The observed differences in sorption to the matrix as well as partitioning to the acceptor fluid indicate that significant differences among terpenes exist. Lipophilicities of the terpenes were not found in the literature and they were calculated using the ACD/ChemSketch v4.55 program (Advanced Chemistry Development, Canada). The calculated $\log P$ values were as follows: 4.67(±0.24) for terpinolene, 4.58(±0.24) for limonene and 2.82(±0.27) for eucalyptol. Thus, larger release of eucalyptol to the aqueous medium may be explained by lower lipophilicity in comparison to other terpenes. However, the reason for the fastest diffusion of limonene in the mixture of D- and L-stereoisomers (dipenetene) is not understood.

The analysis of the curves presented in Fig. 2 reveals two-phase kinetics of the release process. In the first step, up to ~2 h the release is slow and probably corresponds to the surface release of terpenes dissolved in the matrix, while the second phase indicates much faster release of the terpenes from microcompartments formed by pure terpene in the matrix. The T_{lag} of the second phase is longer than 1 h and for two terpenes, namely D-limonene and terpinolene, even longer than 2 h. The matrices do not swell in water and do not absorb water, and such behaviour is a reason for the delayed release process. Despite the significant decrease in the content of terpenes in matrices (e.g. for dipenetene by 41.4%) a constant release rate was observed for 8 h, which may also indicate that the release occurs from the microcompartments rather than from the matrix.

When dermis was a barrier, the kinetics of penetration to the buffer media was very similar to the one observed for the release process directly to the acceptor media (Table 2; Fig. 3). Neither flux nor T_{lag} were affected by the presence of dermis. Only for L-limonene did the flux decrease, but the difference, although statistically significant, was small. Finally, a linear relationship between the amount of terpene penetrating through dermis and the square root of time was observed (Fig. 3). This allows us to conclude that dermis does not present a barrier in the diffusion of terpenes.

Penetration profiles of terpenes through the epidermis with intact stratum corneum are presented in Fig. 4. A relatively short T_{lag}, approximately 2 h, is characteristic for all terpenes and indicates fast diffusion. For terpinolene and D-limonene it is even shorter than observed in the experiments without the barrier, which should be rather related to a non-uniform distribution of the terpenes in the matrix. The rate of penetration was slower in the presence of epidermis for all terpenes, proving that this layer is a barrier. Significant differences in the permeability of terpenes are observed (Fig. 4; Table 2). Penetration of dipenetene, for example, is only slightly affected by the presence of the epidermal barrier and is the fastest (K_p is 1.8×10^{-3} cm/h). On the other hand, when the isomers are applied separately, the resulting K_p values are 3.3 and 8.6 times smaller, for D-limonene and L-limonene, respectively. Such

an effect can only be partly explained by different initial concentrations. Higher loading of dipenetene in the matrix and faster penetration through epidermis may indicate that the mixture of stereoisomers presents a synergistic effect, but such a hypothesis requires further investigation. The unique character of dipenetene was also exhibited in the macroscopic observation of the skin, which was swollen and corrosively damaged after prolonged contact with this terpene under occlusive conditions. These changes were not observed in the skin treated by other terpenes.

The amounts of terpenes penetrating through the skin are in agreement with data published for other terpenes. Similar amounts of L-menthol ($\sim$ 0.8 mg/cm^2) penetrated after 8 h from an aqueous-ethanolic solution through human skin in vitro (Sugibayashi et al., 1995). In our previous in vitro studies with matrices composed of polyacrylate polymer and macrogol, similar penetration of terpinolene through human dermis ($\sim$ 1.3 mg/cm^2) or stratum corneum ($\sim$ 0.7 mg/cm^2) was determined (Janicki et al., 2000). It may be possible that the penetration of terpenes does not significantly depend on the kind of carrier (if it does not contain ethanol as co-enhancer), being limited only by stratum corneum.

Fig. 5 shows the amounts of terpenes found in the skin layers. When the difference in thickness of epidermis and dermis is taken into consideration, the higher affinity of terpenes to epidermis than dermis can be demonstrated. The dry mass of epidermis is approximately 2–3 mg/cm^2, thus the amounts of terpenes found in this layer correspond to over 50% of the total mass. Such a good partitioning of terpenes to epidermis, most probably to stratum corneum, is a reason for good penetration through the skin. Binding of eucalyptol to the tissue cannot be excluded, since skin accumulation of this terpene is nearly two times larger than dipenetene, despite the similar fluxes.

References

Cal, K., Gabiga, H., Kwiatkowski, B., Janicki, S., 1997. Comparative studies of transdermal penetration of propylene glycol and oleic acid in rats from the formulation containing various drugs. Farm. Pol. 53, 200–203 In Polish.

Ceschel, G.C., Maffei, P., Moretti, M.D.L., Demontis, S., Peana, A.T., 2000. In vitro permeation through porcine buccal mucosa of *Salvia desoleana* Atzei and Picci essential oil from topical formulations. Int. J. Pharm. 195, 171–177.

Cornwell, P.A., Barry, B.W., Bouwstra, J.A., Gooris, G.S., 1996. Modes of action of terpene penetration enhancers in human skin; differential scanning calorimetry, small-angle X-ray diffraction and enhancer uptake studies. Int. J. Pharm. 127, 9–26.

Gabiga, H., Cal, K., Kwiatkowski, B., Janicki, S., 1996. In vitro percutaneous penetration of sorption promoters: oleic acid and glyceryl trioleate. Farm. Pol. 52, 435–439 In Polish.

Gabiga, H., Cal, K., Kwiatkowski, B., Janicki, S., Brzoskowska-Paterska, M., 1997. Comparative in vitro studies of percutaneous penetration of propylene glycol and oleic acid in the presence of some drugs in the formulation. Farm. Pol. 53, 195–199 In Polish.

Gabiga, H., Cal, K., Janicki, S., 2000. Effect of penetration enhancers on isosorbide dinitrate penetration through rat skin from a transdermal therapeutic system. Int. J. Pharm. 199, 1–6.

Gao, S., Singh, J., 1997. Mechanism of transdermal transport of 5-fluorouracil by terpenes: carvone, 1,8-cineole and thymol. Int. J. Pharm. 154, 67–77.

Janicki, S., Cal, K., Pocwiardowska, A., 2000. Effect of matrix composition on the in vitro penetration of indomethacin from transdermal therapeutic system through human skin. Farm. Pol. 56, 111–116 In Polish.

Katayama, K., Takahashi, O., Matsui, R., Morigaki, S., Aiba, T., Kakemi, M., Koizumi, T., 1992. Effect of L-menthol on the permeation of indomethacin, mannitol and cortisone through excised hairless mouse skin. Chem. Pharm. Bull. 40, 3097–3099.

Kligman, A.M., Christophers, E., 1963. Preparation of isolated sheets of human stratum corneum. Arch. Dermatol. 88, 702–705.

Kobayashi, D., Matsuzawa, T., Sugibayashi, K., Morimoto, Y., Kobayashi, M., Kimura, M., 1993. Feasibility of use of several cardiovascular agents in transdermal therapeutic systems with L-menthol–ethanol system on hairless rat and human skin. Biol. Pharm. Bull. 16, 254–258.

Moghimi, H.R., Williams, A.C., Barry, B.W., 1996. A lamellar matrix model for stratum corneum intercellular lipids. III. Effects of terpene penetration enhancers on the release of 5-fluorouracil and oestradiol from the matrix. Int. J. Pharm. 145, 37–47.

Moghimi, H.R., Williams, A.C., Barry, B.W., 1997. A lamellar matrix model for stratum corneum intercellular lipids. V. Effects of terpene penetration enhancers on the structure and thermal behaviour of the matrix. Int. J. Pharm. 146, 41–54.

Obata, Y., Ozawa, K., Takayama, K., Nagai, T., 1999. Effect of terpenes on skin permeation of non-ionized and ionized ketotifen. S. T. P. Pharma 9, 497–503.

Sugibayashi, K., Kobayashi, D., Nakagaki, E., Hatanaka, T., Inoue, N., Kusumi, S., Kobayashi, M., Kimura, M., Morimoto, Y., 1995. Differences in enhancing effect of L-menthol, ethanol and their combination between hairless rat and human skin. Int. J. Pharm. 113, 189–197.

Sznitowska, M., Janicki, S., Williams, A.C., 1998. Intracellular or intercellular localization of the polar pathway of penetration across stratum corneum. J. Pharm. Sci. 87, 1109–1114.

Williams, A.C., Barry, B.W., 1991. Terpenes and the lipid–protein-partitioning theory of skin penetration enhancement. Pharm. Res. 8, 17–24.

Williams, A.C., Barry, B.W., 1992. Skin absorption enhancers. Crit. Rev. Therap Drug Carrier Syst. 9, 305–353.