Drug Development and Industrial Pharmacy, 33:1169–1175, 2007

ISSN: 0363-9045 print / 1520-5762 online
DOI: 10.1080/03639040701199258

informa
healthcare

Conjugates of Unsaturated Fatty Acids with Propylene Glycol as Potentially Less-Irritant Skin Penetration Enhancers

Shimon Ben-Shabat, Nir Baruch, and Amnon C. Sintov

Department of Pharmacology and School of Pharmacy, E.D. Bergmann Campus, Ben-Gurion University of the Negev, Beer Sheva, Israel

Fatty acids (FA) are well known as efficient enhancers for transdermal delivery of drugs; however, their frequent dermal toxicity limits their regular use. In order to utilize the fatty acid as a safe enhancer devoid of its irritant effect, we have synthesized and evaluated a series of fatty acids conjugated to propylene glycol (FA-PG). Each one of the conjugates was prepared as a mono- or di- acyl ester derivative. The effects of the synthetic enhancers on the porcine skin permeability were evaluated in a diffusion cell system using lidocaine as the model drug. In addition, in vivo examinations in rabbits were preformed for skin toxicological evaluation. The results indicate that among the FA-PG conjugates, oleic acid (C18:1_{n-9})-PG, linoleic acid (C18:2_{n-6})-PG and α-linolenic acid (C18:3_{n-3})-PG, mono- or di-esters, enhance the penetration of lidocaine relatively to the vehicle (without enhancer). The conjugates of oleic acid (C18:1_{n-9}) and linoleic acid (C18:2_{n-6}) with PG have demonstrated a similar enhancing effect as the corresponding free fatty acids. Interestingly, although the mono- or the di- conjugates of α-linolenic acid (C18:3_{n-3}) with PG enhanced the lidocaine flux as the other two fatty acid conjugates, they resulted in a reduced permeability as compared to the action of their free acid. In addition, the mono-conjugates of α-linolenic acid (C18:3_{n-3}) with PG exhibited elevated skin irritation in rabbits (relative to the fatty acid alone) compared to the significantly reduced irritation of oleate-PG and linoeate-PG mono-conjugates. In conclusion, except saturated FA-PG and α-linolenic acid (C18:3_{n-3})—PG mono-conjugates, unsaturated fatty acids (e.g., oleic and linoleic acids) after conjugation to PG may be safe and effective enhancers for delivering topical drugs.

Keywords skin penetration enhancers; percutaneous penetration; skin irritation; polyunsaturated fatty acids; licodaine

INTRODUCTION

Transdermal or topical delivery of most drugs is frequently limited by the poor skin permeability, particularly to hydrophilic and high molecular-weight drugs. The most efficient barrier to drugs and other substances is the outermost layer of the skin, the stratum corneum (SC), which is comprised of keratin-rich cells embedded in multiple lipid bilayers. The formidable barrier properties can often be overcome by the use of penetration enhancers, chemicals that interact with skin constituents to increase drug flux (Aungst et al., 1986; Buyuktimkin et al., 1997; Finnin & Morgan, 1999; Smith & Maibach, 1995; Walters, 1989). However, many chemical enhancers are toxic, skin irritating or allergenic at some degree or another depending on their concentration and the frequency of their treatment. Therefore, the development of new relatively safer enhancers is desirable. It is also important to understand the mechanisms of enhancement and use this knowledge to design safer and more effective penetration enhancers for dermal and transdermal drugs.

Fatty acids have been shown to accelerate skin permeation of drugs (Aungst et al., 1986; Aungst, 1989; Santoyo & Ygartua, 2000; Williams & Barry, 2004), usually when applied in preparations containing propylene glycol as the co-solvent. The most effective among the saturated fatty acids were those with C_{10}–C_{12} chain lengths (e.g., capric and lauric acids), however, unsaturation of C_{18} fatty acids (e.g., oleic and linoleic acids) also appeared to increase the penetration enhancing effects (Aungst et al., 1986; Aungst, 1989). Considerable efforts have been directed at investigating the mechanism of action of the most popular penetration enhancer, oleic acid (*cis*-9-octadecenoic acid). It was proposed that because of its kinked structure (i.e., the bent *cis* configuration), oleic acid disturbs intercellular lipid packing resulting in separation in the SC lipid domains, thereby reducing their barrier function (Loftsson et al., 1995; Naik et al., 1995; Ongpipattanakul et al., 1991). In contrast to the extensive research done with oleic acid, there have been only a few studies using polyunsaturated fatty acids (PUFA) as penetration enhancers. Loftsson et al. (1995, 1998) used a fatty acid extract from medicinal cod-liver oil as an effective transdermal penetration enhancer, and this effect was found to be associated with the unsaturated portion of ω-3 series of fatty acids. Long-chain fatty acids and other hydrophobic enhancers have usually been applied in combination with a relatively hydrophilic vehicle, such as propylene glycol (PG), to

Address correspondence to Amnon C. Sintov, Department of Pharmacology and School of Pharmacy, E.D. Bergmann Campus, Ben-Gurion University of the Negev, P.O. Box 653, 84105 Beer Sheva, Israel. E-mail: asintov@bgu.ac.il

achieve an augmented accelerant effect. One possible explanation for this augmented effect is the facilitated incorporation of a fatty acid (i.e., oleic acid) into the stratum corneum lipid alkyl domain by the interaction of PG at the polar head group region (Oh et al., 1998). Even when PG is applied without fatty acids, it is known as an efficient co-solvent because it can alter the skin structure, thereby modifying the percutaneous absorption (Bendas et al., 1995; Okamoto et al., 1990). It also readily permeates the skin during which functioning as a carrier for the drug molecules (Squillante et al., 1998). In addition, it has been shown that PG in combination with isopropyl myristate resulted in a synergistic enhancement (Arellano et al., 1999). Anyway, although several pharmaceutical preparations contain unsaturated fatty acids (e.g., oleic acid) and PG, the general use of this combination is limited due to the high prevalence of dermal side effects including SC lipid extraction and damage to viable epidermal cells, which presumably occurs because of the acidic nature of these fatty acids (Sintov et al., 1999; Touitou et al., 2002).

To utilize long-chain unsaturated fatty acids as safe enhancers devoid of their irritant effect, there is a need to modify the carboxylic terminal thus minimizing their acidic properties. Therefore, based on the traditional combination of PG and fatty acids, we have synthesized a series of unsaturated fatty acids conjugated to propylene glycol through ester bonds. We also evaluated the skin penetration enhancement effects of these new conjugates in vitro using porcine skin. The effects of the synthetic ester conjugates as penetration enhancers were evaluated by using lidocaine as a model drug. In addition, any possible damage to the skin was examined in a primary skin irritation test in rabbits.

MATERIALS AND METHODS

Materials

Lidocaine (as base), propylene glycol (PG), tetraglycol as well as the following fatty acids: stearic acid (18:0), oleic acid ($18:1_{n-9}$), linoleic acid ($18:2_{n-6}$), α-linolenic acid ($18:3_{n-3}$), and γ-linolenic acid ($18:3_{n-6}$) were purchased from Sigma, Rehovot, Israel. The reagents, dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)-pyridine (DMAP) were also obtained from Sigma, Rehovot, Israel. High-performance liquid chromatography (HPLC) grade solvents were obtained from J.T. Baker (Mallinckrodt Baker, Inc., Phillipsburg, NJ).

General Method for Synthesis of Conjugates

Dicyclohexylcarbodiimide (DCC) (0.25 mmol) and 4-(dimethylamino)-pyridine (DMAP) (0.025 mmol) were added into a solution of propylene glycol (0.25 mmol) in dry CH_2Cl_2 (20 ml). The mixture was stirred at 0°C, and a solution of stearic acid, oleic acid, linoleic acid, α-linolenic acid or γ-linolenic acid (0.25 mmol) in dry CH_2Cl_2 (2 ml) was added dropwise under nitrogen atmosphere. The mixture was stirred at room temperature for 2 hr. The reaction mixture was washed with 0.5N HCl, saturated $NaHCO_3$ solution, water, and then dried (with $MgSO_4$). The solvent was evaporated under reduced pressure, and the residue was loaded onto a silica gel column (ca. 5g), and the compounds were separated with solvent system of 5% ethyl ether in petroleum ether or 1% ethyl ether in hexane (for stearic acid-propylene glycol conjugate only) to give the mono- and di-ester derivatives of the conjugates. The fatty acid derivatives were routinely stored at −20°C until use.

NMR Spectra: ^{1}H NMR spectra were recorded on a Bruker DMX-500 operating at 500.1 MHz, and chemical shifts are reported in parts per million (δ) using TMS as the internal standard. ***Mono-Stearic acid—PG:*** ^{1}H-NMR ($CDCl_3$): δ 4.03 (m, 1H), 3.65 (d, 2H), 2.26 (m, 2H), 1.68 (br m, 2H), 1.54 (s, 3H), 1.24 (br m, 2H), 1.124–1.183 (m, 26H), 0.823 (t, 3H). ***Di-Stearic acid—PG:*** ^{1}H-NMR ($CDCl_3$): δ 5.06 (m, 1H), 4.03 (m, 2H), 2.24 (m, 4H), 2.10 (s, 4H), 1.23 (m, 3H), 1.12–1.18 (m, 56H), 0.84 (m, 6H). ***Mono-Oleic acid—PG:*** ^{1}H-NMR ($CDCl_3$): δ 5.28 (br s, 2H), 4.05 (m, 1H), 3.85 (m, 2H), 2.32 (m, 2H) 2.28 (m, 4H), 1.94 (m, 2H), 1.42 (s, 3H), 1.33 (m, 6H), 1.23 (m, 14H), 0.81 (t, 3H). Di-Oleic acid—PG: ^{1}H-NMR($CDCl_3$): δ 5.28 (m, (4H), 5.07 (m, 1H), 3.98 (m, 2H), 2.23 (m, 4H), 2.09 (s, 8H), 1.93 (m, 4H), 1.57 (m, 3H), 1.25 (m, 12H), 1.13–1.23 (m, 28H), 0.85 (m, 6H). ***Mono-Linoleic acid—PG:*** ^{1}H-NMR ($CDCl_3$): δ 5.27–5.31 (m, 4H), 4.02 (m, 1H), 3.80 (m, 2H), 2.68 (t, 2H), 2.279 (m, 2H), 1.94 (q, 4H), 1.58 (m, 2H), 1.28 (br s, 3H), 1.12–1.17 (m, 14H), 0.833 (t, 3H). ***Di-Linoleic acid—PG:*** ^{1}H-NMR ($CDCl_3$): δ 5.25–5.31 (m, 8H), 5.08 (m, 1H), 3.99 (m, 2H), 2.71 (t, 4H), 2.23 (m, 4H), 2.11 (br s, 8H), 1.97 (q, 4H), 1.54 (br s, 3H), 1.29 (m, 12H), 1.23 (br s, 16H), 0.85 (m, 6H). ***Mono-Linolenic acid—PG:*** ^{1}H-NMR ($CDCl_3$): δ5.27–5.30 (m, 6H), 4.152 (m, 1H), 3.635 (m, 2H), 2.74(br m, 4H), 2.338 (t, 2H), 1.99 (m, 4H), 1.62 (m, 2H), 1.42 (m, 3H), 1.17–1.29 (m, 8H), 0.92 (t, 3H). ***Di-Linolenic acid—PG:*** ^{1}H-NMR ($CDCl_3$): δ5.26–5.35 (m, 12H), 5.01 (m, 1H), 4.32 (m, 2H), 2.73 (m, 8H), 2.22 (m, 4H), 2.11 (br s, 8H), 1.97 (q, 4H), 1.54 (br s, 3H), 1.29 (m, 16H), 0.83 (m, 6H). ***Mono-γ-Linolenic acid—PG:*** ^{1}H-NMR ($CDCl_3$): δ5.27–5.31 (m, 6H), 4.08 (m, 1H), 3.75 (m, 2H), 2.74(t, 4H), 2.35 (t, 2H), 2.01 (m, 4H), 1.64 (m, 2H), 1.36 (m, 3H), 1.16–1.28 (m, 8H), 0.83 (t, 3H). ***Di-γ-Linolenic acid—PG:*** ^{1}H-NMR ($CDCl_3$): δ5.28–5.30 (m, 12H), 5.04 (m, 1H), 4.23 (m, 2H), 2.72 (m, 8H), 2.22 (m, 4H), 2.11 (br s, 8H), 1.97 (q, 4H), 1.54 (m, 3H), 1.28 (m, 16H), 0.82 (m, 6H). MS spectra were carried out using an Agilent 1100LC series (Waldbronn, Germany) and Bruker Esquire 3000plus MS (Bremen, Germany) instrument. ESI-MS-API electron spray interface, positive mode polarity measurements were preformed to obtain the $[M+Na]^+$ molecular weights.

In Vitro Skin Permeation Study

The permeability of lidocaine through porcine skin was determined in vitro with a Franz diffusion cell system (Crown Bioscientific, Inc., Clinton, NJ). The diffusion area was 1.767 cm^2 (15 mm diameter orifice), and the receptor compartment volumes varied from 11 to 12 ml. The solutions in the receiver side were

stirred by externally driven, Teflon-coated magnetic bars. The testing was basically performed as previously described (Ben Shabat et al., 2005; Sintov & Botner, 2006a, b; Sintov & Shapiro, 2004). Each set of experiments was performed with at least four diffusion cells ($n \geq 4$). Full-thickness porcine skin was excised from fresh ears of slaughtered white pigs (breeding of Landres and Large White, locally grown in The Institute of Animal Research, Kibbutz Lahav, Israel), and the hair was trimmed off. Skin sections (about 2×2 cm) were excised, and subcutaneous fat was removed with a scalpel. Transepidermal water loss (TEWL) measurements (Dermalab® Cortex Technology, Hadsund, Denmark) were performed on all pieces, and only those for which TEWL levels fell within specification (<15 g/m^{2}hr) were mounted in the diffusion cells, ready for testing. The skin pieces were kept at –20°C and were used for penetration studies within 2 weeks after slaughter of the animal. Each skin section was placed with the stratum corneum facing up on the receiver compartments, and the donor compartments were then clamped in place. The receiver compartment, defined as the side facing the dermis, was filled with phosphate buffer (4 mM, pH 7.4)—ethyl alcohol (analytical grade) (7:3 v/v). The epidermis side was washed with water and dried gently with cotton balls. After 15 min of washing at 37°C, the receiver medium was discarded and the chamber was refilled with a fresh buffer-ethanol solution. This receiver medium was chosen to provide in vivo equivalent "sink" conditions in the receiver compartment, i.e., to increase the circulation in the dermis and to prevent possible sedimentation of permeants in the dermis stagnant layer of the full-thickness skin (Sintov et al., 2002). Aliquots (200 µl per cell) of lidocaine 1% (w/w) solution of tetraglycol-distilled water (1:1 w/w) with or without an enhancer were applied on the skin covering the entire diffusion area. It should be noted that the 1% lidocaine concentration in the vehicle solution is sub-saturated while solubility of lidocaine in this vehicle can reach 12%. Each mono- or di- fatty acid conjugate of PG was dissolved in the lidocaine solution at a concentration that equated 1% w/w of its corresponding free fatty acid on a molar basis. After application, samples (2 ml) were withdrawn from the receiver solution every hour for a period of 8 hr, and the cells were replenished up to their marked volumes with fresh buffer-ethanol solution each time. The samples were taken into 1.5 ml amber vials, and stored at –20°C until analyzed by HPLC.

HPLC Analysis of Samples from Receiver Solutions

Aliquots of 20 µl from each vial were injected onto the HPLC system, equipped with a prepacked CN column (Nucleosil 100-5 CN, 5µm, 250×4 mm, Macherey-Nagel, Dueren, Germany). The HPLC system (Shimadzu VP series) consisted of an autosampler and a diode array detector. The quantitation of lidocaine was performed by integration of the peaks detected at 210 nm. The samples were chromatographed using an isocratic mobile phase consisting 0.02M phosphate buffer pH 6.0—acetonitrile (4:6 v/v) at a flow rate of 0.8 ml·min^{-1}. A calibration curve (peak area versus drug concentration) was constructed by running standard lidocaine solutions in a phosphate buffer/ethanol (7:3 v/v) for each series of chromatographed samples. Calibration curves were linear over the range of 0.5–10 µg·ml^{-1}. As a result of the sampling of large volumes from the receiver solution (and the replacement of these amounts with equal volumes of buffer), the receiver solution was constantly being diluted. Taking this process into account, the cumulative drug permeation (Q_t) was calculated from the following equation:

$$Q_t = V_r C_t + \sum_{i=0}^{t-1} V_S C_i$$

where C_t is the drug concentration of the receiver solution at each sampling time, C_i is the drug concentration of the ith sample, and V_r and V_s are the volumes of the receiver solution and the sample, respectively. Data were expressed as the cumulative lidocaine permeation per unit of skin surface area, Q_t/S (S=1.767 cm^2). The steady-state fluxes (J_{ss}) were calculated by linear regression interpolation of the experimental data at a steady state:

$$J_{SS} = \Delta Q_t / (\Delta TS)$$

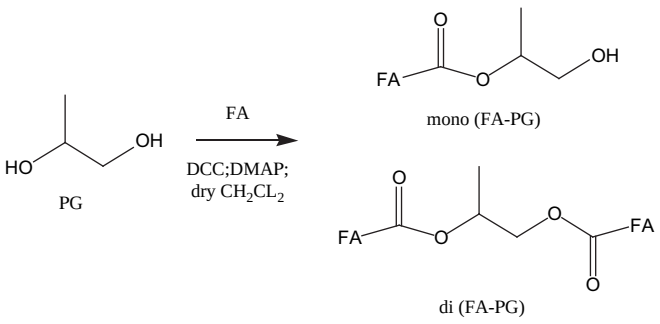
In Vivo Application (Primary Skin Irritation Test)

The animal protocol was reviewed and approved by the institutional Animal Care & Use Committee, which complies with the Israeli Law of Human Care and Use of Laboratory Animals, 1994. Three male albino rabbits weighing approximately between 2.5 and 3.0 kg were used to examine the potential dermal irritation induced by the fatty acids and their PG conjugates. 24 hr before the application of FAs and their conjugates, the dorsal hair was removed by an electric clipper. The clipped area was divided into 12 equal squares. Adhesive plasters having circular holes in the middle (1.5 cm in diameter) were affixed to the dorsal area, one on each square. Eight lidocaine 1% (w/w) test solutions (100 µl) of tetraglycol-distilled water (1:1 w/w) with a conjugate or with its corresponding FA were applied on the skin in each circular area. The eight tested enhancers were stearic acid (18:0), oleic acid (18:1$_{n-9}$), linoleic acid (18:2$_{n-6}$), α-linolenic acid (18:3$_{n-3}$), and their corresponding mono-conjugates. The application of the test solutions was performed in a random order on the left and the right sides of the back (each side was divided into 4 squares), while one of the middle squares was used for application of the vehicle and the other squares were left without treatment. The dorsal zone was covered with sterile pads and occlusive dressings, which were removed for observation after 1, 3, 5, and 7 days. After each observation and assessment, the sterile pads were changed and the animals' backs were reoccluded. Erythema and edema were evaluated for each site according to the modification of Draize's method and graded from 0 to 8 (Sasaki et al., 1990). The primary skin irritation test index (PIT) was calculated from adding up all the scores and dividing by the number of animals.

RESULTS AND DISCUSSION

New conjugates built of propylene glycol and fatty acids were prepared. We involved in the reactions a saturated fatty acid, stearic acid (C18:0), and an unsaturated fatty acid, oleic acid (C18:1_{n-9}). In addition, three polyunsaturated fatty acids (PUFA)—linoleic acid (C18:2_{n-6}), linolenic acid (C18:3_{n-3}) and γ-linolenic acid (C18:3_{n-6})—were used. Each fatty acid (FA) was condensed with propylene glycol (PG) in the presence of dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)-pyridine (DMAP) in dry dichloromethane to give the relevant conjugates, the monoester derivative and the diester derivative, in which one or two acyl groups were attached to one or two hydroxyl terminals of PG, respectively (Scheme 1). Two isomers of the monoFA-PG conjugate were obtained, however, the preferable mono-isomer during synthesis was found to be the one where the methyl group of PG was at the α position to the ester bond so that the skin penetration evaluation was performed with this isomer only (Scheme 1).

Figure 1 exemplifies the kinetic profiles of lidocaine penetration through porcine skin using 1% preparations with or without the new monoester enhancers. For all monoester and diester conjugates as well as for the free fatty acids, a similar pattern was observed. After lag time period, which lasted between 2 and 3 hr, a linear steady state flux was obtained throughout the 8 h experiments. Table 1 summarizes the results and Figure 2 illustrates the various fluxes obtained by using the different fatty acids, their mono- and di-esters. It has been shown that the conjugates of oleic acid-PG, linoleic acid-PG, and linolenic acid-PG, mono- or di-ester derivatives, enhanced the penetration of lidocaine relatively to the vehicle alone. It is interesting to note that the monoester and the diester conjugates of oleic acid (18:1_{n-9}) and linoleic acid (18:2_{n-6}) accelerated the percutaneous penetration flux of lidocaine to a similar degree as the flux obtained by the corresponding free fatty acid. The enhancement ratios obtained by oleic and linoleic acids (or their conjugates) were approximately 2 and 1.5, respectively. It can be concluded that lidocaine penetration enhancement effect of oleic and linoleic acids was not influenced neither by the addition of PG moiety to one fatty acid molecule nor by linking two molecules of these fatty acids. In



SCHEME 1. Condensation reaction of fatty acid (FA) with propylene glycol (PG).

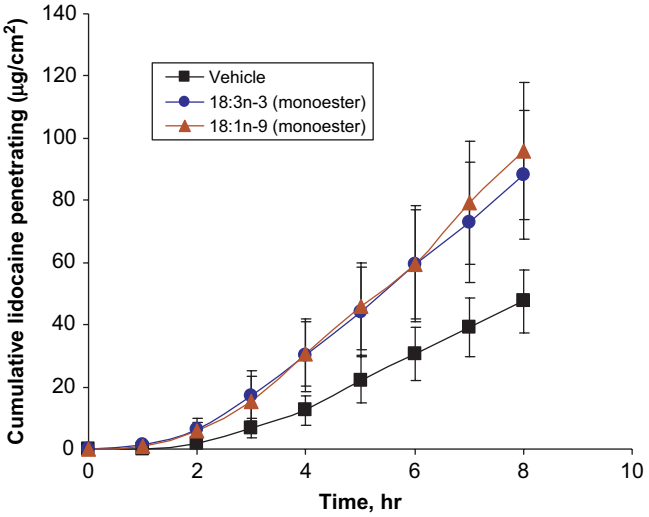


FIGURE 1. Percutaneous permeation kinetic profiles of lidocaine (1% preparation) without enhancer (■), with PG monoester of linolenic acid (•) and with PG monoester of oleic acid (▲).

contrast, the monoester and the diester of linolenic acid (18:3_{n-3}) resulted in relatively lower lidocaine fluxes (the enhancement ratios were 1.7 and 1.4, respectively) as compared to the penetration flux obtained by the free linolenic acid (enhancement ratio = 3.1) ($p < 0.05$). This interesting phenomenon might be explained in terms of partial ionization of the free carboxyl group of linolenic acid. It was noted in a previous publication that ionized oleic acid in propylene glycol/water mixture accelerated drug penetration and skin irritancy more than the unionized form of the fatty acid (Sintov et al., 1999). Another report has pointed out that linolenic acid possesses a pK_a of 8.28, which is considerably lower than oleic and linoleic acids (9.85 and 9.24, respectively), indicating a presence of more ionized molecules at physiological pH (Kanicky & Shah, 2002). This is actually not surprising if the steric configuration of linolenic acid is taken into consideration. As shown in Figure 3, the chain of linolenic acid contains kinks and bends due to the three *cis* double bonds. Since these kinks prevent the molecules to be packed closely, the molecules can be easily ionized, form ion-dipole interactions with the polyol in the vehicle and penetrates more rapidly than regular non-ionized micelles (Kanicky & Shah, 2002; Sintov et al., 1999). In the case of linolenic acid conjugates, in which the carboxylic polar groups were replaced by the non-ionizable propylene glycol moieties, similar penetration fluxes of lidocaine were obtained as provided by oleic and linoleic acids or their conjugates (Table 1). The enhancement effects of oleic acid, linoleic acid and linolenic acid, as demonstrated in our research for lidocaine delivery, are in accordance with several previous studies showing similar enhancement for naloxone, piroxicam and melatonine (Aungst, 1989; Kanikkannan et al., 2000; Santoyo & Ygartua, 2000). However, the substantial enhancement effects that were achieved by oleic, linoleic and

TABLE 1
Summary of Skin Penetration Fluxes of 1% (w/w) Lidocaine in Solution Containing Various Fatty Acid-based Enhancers at Equimolar Concentrations (35—36 mM Equivalent to 1% of Each Corresponding Free Fatty Acid)

Enhancer	Flux ($\mu g/cm^2/h$)	Enhancement Ratio	Difference from the Flux Obtained Without Enhancer	Cumulative Amount of Lidocaine Penetrated at t = 8h ($\mu g/cm^2$)
No enhancer	8.38 ± 1.50	1.00	–	47.60 ± 10.04
Stearic acid (18:0)	5.96 ± 1.87	0.71	No	30.53 ± 10.29
PG mono-stearate	7.79 ± 2.08	0.93	No	40.76 ± 11.21
PG di-stearate	7.78 ± 2.31	0.93	No	41.48 ± 13.62
Oleic acid ($18:1_{n-9}$)	17.98 ± 5.22	2.14	***	98.00 ± 27.05
PG mono-oleate	16.04 ± 3.33	1.91	***	95.78 ± 22.15
PG di-oleate	17.68 ± 1.39	2.11	***	96.45 ± 6.70
Linoleic acid ($18:2_{n-6}$)	13.29 ± 2.66	1.58	***	84.78 ± 15.41
PG mono-linoleate	14.08 ± 4.33	1.68	**	78.68 ± 24.92
PG di-linoleate	12.09 ± 1.84	1.44	***	71.52 ± 16.60
Linolenic acid ($18:3_{n-3}$)	26.39 ± 7.08	3.15	***	135.98 ± 30.22
PG mono-linolenate	14.25 ± 2.65	1.70	***	88.17 ± 20.73
PG di-linolenate	11.48 ± 1.33	1.37	*	67.26 ± 10.72
γ-Linolenic acid ($18:3_{n-6}$)	7.75 ± 2.29	0.92	No	44.37 ± 14.19
PG mono-γ-linolenate	9.41 ± 1.99	1.12	No	53.78 ± 11.23
PG di-γ–linolenate	8.55 ± 2.22	1.02	No	50.24 ± 15.46

Note: PG = Propylene glycol. *$p < 0.05$ (student t-test). **$p < 0.01$ (student t-test). ***$p < 0.005$ (student t-test).

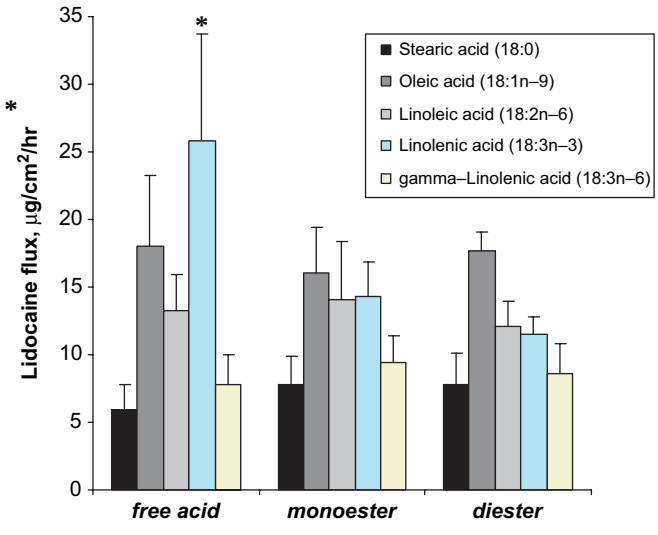


FIGURE 2. Graphic illustration of the mean lidocaine fluxes obtained from different fatty acids and from their corresponding mono- and di-esters of propylene glycol. An asterisk represents statistical significance compared with other forms of the corresponding fatty acid ($p < 0.05$).

linolenic acids and their conjugates were not obtained by the saturated stearic acid, by the polyunsaturated γ-linolenic acid or by their PG conjugates. It has already been shown by Aungst et al. (1986) and by Kanikkannan et al. (2000) that saturated fatty acids with chain lengths higher than 11 had a decreased penetration enhancing effect for naloxone and melatonin. Similarly, the lidocaine penetration flux obtained in our studies was not enhanced by stearic acid or its esters. The fact that the polyunsaturated γ-linolenic acid did not increase the penetration flux of lidocaine as did α-linolenic acid can be easily explained by the geometric variation of these isomers. As illustrated in Figure 3, γ-linolenic acid possesses dominantly a globular structure while α-linolenic acid exhibits a more stretched-chain form. As established by the accumulated evidence to date on the mechanism of action of the enhancers, they intercalate/partition into the relatively highly ordered region of the lipid bilayers, inducing disorder, and increasing fluidity in this region. Since partitioning into the ordered region could be much easier if the molecules contain straight chains, it may explain why α-linolenic acid and its conjugates would act effectively rather than γ-linolenic acid in fluidizing the lipid bilayers and enhancing the drug penetration flux.

In contrast to the increased penetration enhancement of linolenic acid, indicating an easy disruption of the free acid of the lipid bilayers, the skin irritation test has surprisingly revealed that this acid caused a significantly less erythema of the skin compared to its conjugate, the monoester derivative. As shown in Figure 4c, the monoester of linolenic acid resulted in a severe damage relative to that observed after application of the free acid ($p < 0.05$ by two-way ANOVA test). Unlike the linolenic acid ester derivative, the PG esters of oleic and linoleic acids

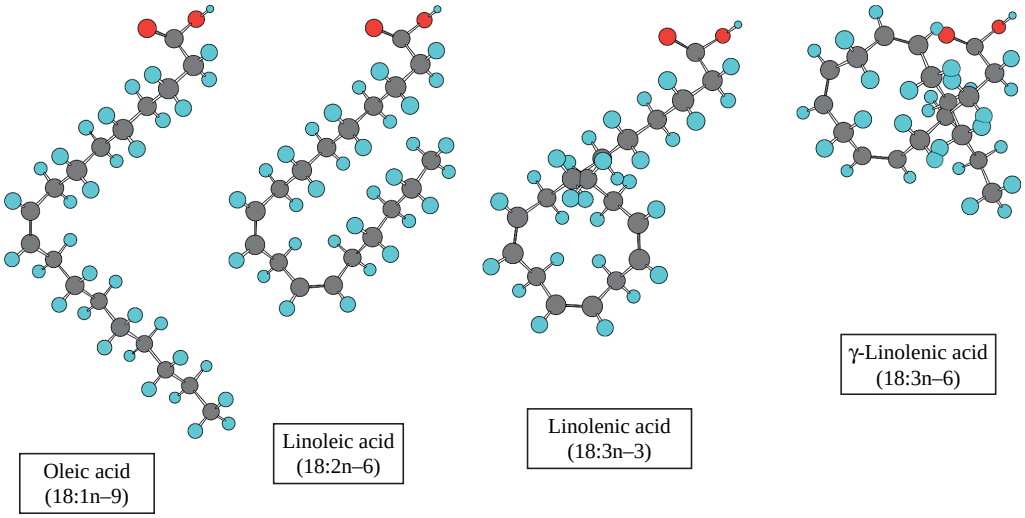


FIGURE 3. Three-dimensional structures of oleic acid, linoleic acid, linolenic and γ-linolenic acids showing the differences in their spatial configuration.

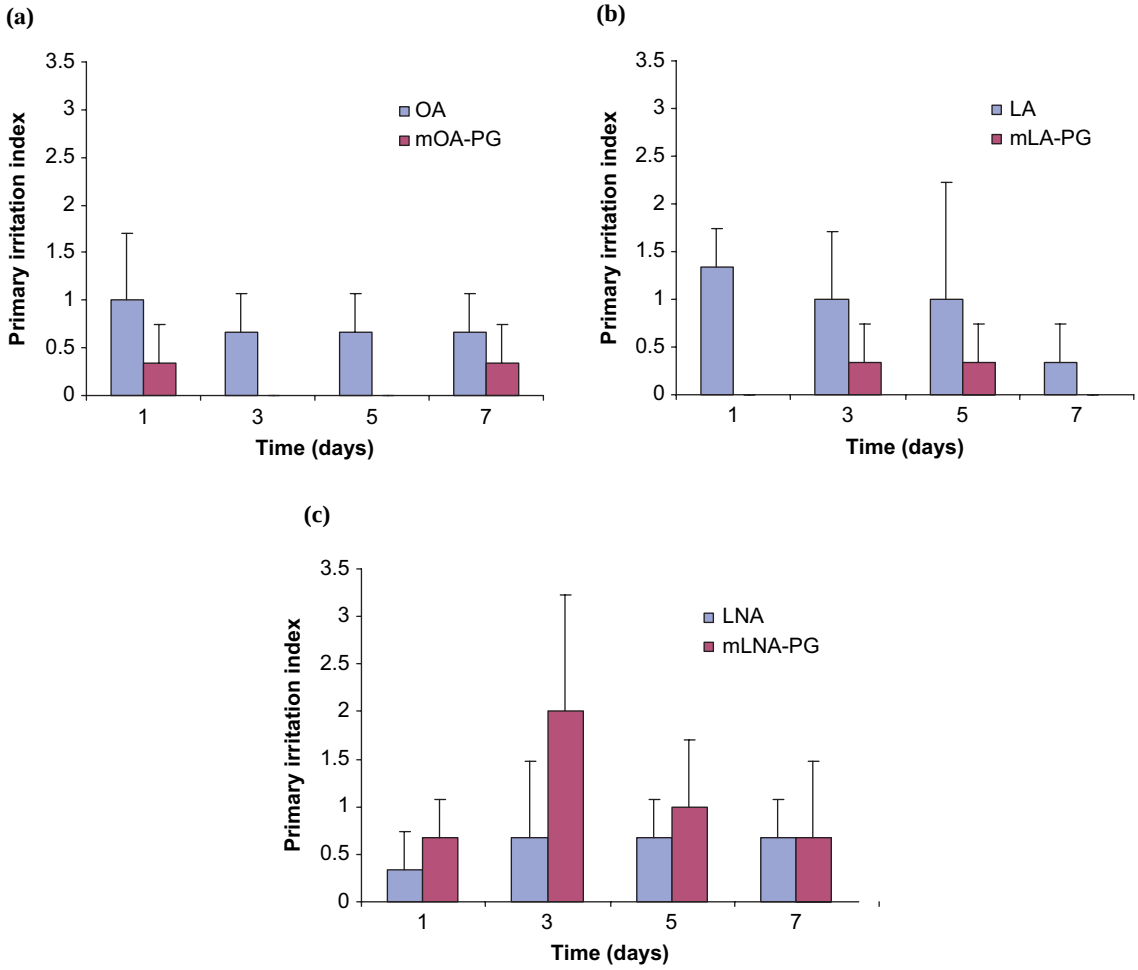


FIGURE 4. Mean primary skin irritation indices (± *SE*) obtained during 7-day period using the rabbit model ($n = 3$). The enhancer's test solutions were applied in a random order on the shaved back of each rabbit. Erythema/edema scoring was performed on the test sites and compared to vehicle-treated and no-treated sites. OA = oleic acid, mOA-PG = PG mono-oleate, LA = linoleic acid, mLA-PG = PG mono-linoleate, LNA = α-linolenic acid, mLNA-PG = PG mono-linolenate.

resulted in less erythema than the corresponding free acids (Figure 4). Stearic acid and its PG ester showed no irritation to the skin. No edema was noted during the 7 day test after treatment by the free acids as well as after treatment by their esters.

In developing penetration enhancers for topical or transdermal delivery, it is essential to minimize the potential of the enhancer to irritate the skin. As published by numerous researchers, unsaturated fatty acids (e.g., oleic acid) are effective penetration enhancers that increase the permeability of drugs but frequently at the expense of causing irritation. We have made an attempt to reduce the irritant effect of unsaturated fatty acids by their conjugation to propylene glycol to form mono- or di-esters. The results presented in this article show that the conjugation to oleic and linoleic acids, on the one hand, preserves the enhancement effect of the free acids and, on the other hand, diminishes skin irritation during 7-d application period compared with that caused by the free fatty acid. The monoester and the diester of oleic acid or linoleic acid with propylene glycol increased the permeability relatively to the vehicle but at the same degree, as did the free acids. The esters of linolenic acid with propylene glycol increased the penetration of lidocaine at the same degree as did the oleic and the linoleic acid esters, but at a reduced intensity compared to the action of the free linolenic acid. Further investigations are necessary to elucidate the mechanisms of the penetration enhancing effect obtained by the various conjugates and the correlation to their toxicity; however, the present study demonstrates their potential advantage for drug transport. It has been concluded, therefore, that selected FA-PG conjugates might be useful enhancers for the delivery of drugs from topical formulations. However, due to the increased skin toxicity of PG-linolenate as described in this paper, the clinical introduction of such conjugates should be strictly regulated by safety studies.

ACKNOWLEDGMENTS

This work was performed in partial fulfillment of the requirements for the M.Sc. degree of N. Baruch's. The authors are grateful for the practical and professional assistance of Mr. Igor Krymberk, Ms. Lillia Shapiro, and Ms. Clara Behar-Canetti.

REFERENCES

Arellano, A., Santoyo, S., Martín, C., & Ygartua, P. (1999). Influence of propylene glycol and isopropyl myristate on the in vitro percutaneous penetration of diclofenac sodium from carbopol gels. *Eur. J. Pharm. Sci., 7,* 129–135.

Aungst, B. J. (1989). Structure/effect studies of fatty acid isomers as skin penetration enhancers and skin irritants. *Pharm. Res., 6,* 244–247.

Aungst, B. J., Rogers, N. J., & Shefter, E. (1986). Enhancement of naloxone penetration through human skin in vitro using fatty acids, fatty alcohols, surfactants, sulfoxides, and amides. *Int. J. Pharm., 33,* 225–234.

Bendas, B., Schmalfuss, U., & Neubert, R. (1995). Influence of propylene glycol as cosolvent on mechanisms of drug transport from hydrogels. *Int. J. Pharm., 116,* 19–30.

Ben-Shabat, S., Benisty, R., Wormser, U., & Sintov, A. C. (2005). Vitamin D_3-based conjugates for topical treatment of psoriasis: Synthesis, antiproliferative activity, and cutaneous penetration studies. *Pharm. Res., 22,* 50–57.

Buyuktimkin, N., Buyuktimkin, S., & Rytting, J. H. (1997). Chemical means of transdermal drug permeation enhancement. In T. K. Ghosh, W.R. Pfister, & S.I. Yum (Eds.), *Transdermal and topical drug delivery systems* (pp. 357–475). IL: Interpharm Press, Inc., Buffalo Grove.

Finnin, B. C., & Morgan, T. M. (1999). Transdermal penetration enhancers: Application, limitation, and potential. *J. Pharm. Sci., 88,* 955–958

Kanicky, J. R., & Shah, D. O. (2002). Effect of degree, type, and position of unsaturation on the pKa of long-chain fatty acids. *J. Colloid Interface. Sci., 256,* 201–207.

Kanikkannan, N., Kandimalla, K., Lamba, S. S., Singh, M. (2000). Structure-activity relationship of chemical penetration enhancers in transdermal drug delivery. *Curr. Med. Chem., 7,* 593–608.

Loftsson, T., Gudmundsdóttir, T. K., Fridriksdóttir, H., Sigurdaróttir, A. M., Thorkelsson, J., Gudmundsson, G., & Hjaltason, B. (1995). Fatty acids from cod-liver oil as skin penetration enhancers. *Pharmazie, 50,* 188–190.

Loftsson, T., Thormar, H., Ólafsson, J. H., Gunnarsdóttir, T. M., Hjaltason, B., & Gudmundsson, G. (1998). Fatty acid extract from cod-liver oil: Activity against herpes simplex virus and enhancement of transdermal delivery of acyclovir in-vitro. *Pharm. Pharmacol. Commun., 4,* 287–291.

Naik, A., Pechtold, L. A. R. M., Potts, R. O., & Guy, R. H. (1995). Mechanism of oleic acid-induced skin penetration enhancement in vivo in humans. *J. Control. Release, 37,* 299–306.

Oh, S. Y., Jeong, S. Y., Park, T. G., & Lee, J. H. (1998). Enhanced transdermal delivery of AZT (zidovudine) using iontophoresis and penetration enhancer. *J. Control. Release, 51,* 161–168.

Okamoto, H., Muta, K., Hashida, M., & Sezaki, H. (1990). Percutaneous penetration of acyclovir through excised hairless mouse and rat skin; Effect of vehicle and percutaneous penetration enhancer. *Pharm. Res., 7,* 64–68.

Ongpipattanakul, B., Burnette, R. R., Potts, R. O., & Francoeur M. L. (1991). Evidence that oleic acid exists in a separate phase within stratum corneum lipids. *Pharm. Res., 8,* 350–354.

Santoyo, S., & Ygartua, P. (2000). Effect of skin pretreatment with fatty acids on percutaneous absorption and skin retention of piroxicam after its topical application. *Eur. J. Pharm. Biopharm., 50,* 245–250.

Sasaki, H., Kojima, M., Nakamura, J., & Shibasaki, J. (1990). Acute toxicity and skin irritation of pyrrolidone derivatives as transdermal penetration enhancer. *Chem. Pharm. Bull., 38,* 2308–2310.

Sintov, A., Ze'evi, A., Uzan, R., & Nyska, A. (1999). Influence of pharmaceutical gel vehicles containing oleic/sodium oleate combinations on hairless mouse skin, a histological evaluation. *Eur. J. Pharm. Biopharm., 47,* 299–303.

Sintov, A. C., Behar-Canetti, C., Friedman, Y., & Tamarkin, D. (2002). Percutaneous penetration and skin metabolism of ethylsalicylate-containing agent, TU-2100: in vitro and in vivo evaluation in guinea pigs. *J. Control. Rel., 79,* 113–122.

Sintov, A. C., & Botner, S. (2006a). Transdermal drug delivery using microemulsion and aqueous systems: influence of skin storage conditions on the in vitro permeability of diclofenac from aqueous vehicle systems. *Int. J. Pharm., 311,* 55–62.

Sintov, A. C., & Brandys-Sitton, R. (2006b). Facilitated skin penetration of lidocaine by using a combination of iontophoresis and microemulsion-based formulation. *Int. J. Pharm., 19,* 58–67.

Sintov, A. C., & Shapiro, L. (2004). New microemulsion vehicle facilitates percutaneous penetration in vitro and cutaneous drug bioavailability in vivo. *J. Control. Rel., 95,* 173–183.

Smith, E. W., & Maibach, H. I. (1995). *Percutaneous penetration enhancers.* FL: CRC Press, Boca Raton.

Squillante, E., Needham, T., Maniar, A., Kislalioglu, S., & Zia, H. (1998). Codiffusion of propylene glycol and dimethyl isosorbide in hairless mouse skin. *Eur. J. Pharm. Biopharm., 46,* 265–271.

Touitou, E., Godin, B., Karl, Y., Bujanover, S., & Becker, Y. (2002). Oleic acid, a skin penetration enhancer, affects Langerhans cells and corneocytes. *J. Control. Release, 80,* 1–7.

Walters, K. A. (1989). Penetration enhancers and their use in transdermal therapeutic systems. In J. Hadgraft, R. H. Guy (Eds.), *Transdermal drug delivery, developmental issues and research initiatives* (pp. 197–246). New York: Marcel Dekker, Inc.

Williams, A. C., & Barry, B. W. (2004). Penetration enhancers. *Adv. Drug Del. Rev., 56,* 603–618.