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In-vitro and in-vivo evaluation of oligoethylene esters as dermal prodrugs of 18β-glycyrrhetic acid

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Abstract

Novel polyoxyethylene esters of 18 β-glycyrrhetic acid (GA) were synthesized and evaluated as potential dermal prodrugs. The permeation of these prodrugs ($\mathbf{1_{a-e}}$) was studied in-vitro, using excised human skin membranes (SCE; stratum corneum/epidermis) mounted in Franz type cells, and in-vivo, evaluating the ability of these compounds to inhibit methyl nicotinate (MN)-induced skin erythema in healthy human subjects. All the esters synthesized showed a good water stability, while the enzymatic hydrolysis rate was significantly affected by the length of the polyoxyethylenic chain used as promoiety. In in-vitro percutaneous absorption studies, only esters $\mathbf{1_b}$ and $\mathbf{1_c}$ (respectively triethylen- and tetraethylenglycol derivatives) showed an increased flux through SCE membranes compared with GA. Furthermore, we observed an appreciable and sustained in-vivo topical anti-inflammatory activity of esters $\mathbf{1_b}$ and $\mathbf{1_c}$ compared with the parent drug.

Introduction

Liquorice, originating from the roots of various species of *Glycyrrhiza* (family Leguminosae) primarily *Glycyrrhiza glabra* L., has been used for medicinal purposes for more than 1000 years. The biological properties of this plant are associated with the presence of glycyrrhizin, a 3-diglucuronide derivative and with its aglycone, 18β-glycyrrhetic acid (GA). GA and its derivatives are mainly used in pharmaceutical and cosmetic fields as lenitive and anti-reddening agents (Capella & Finzi 2003) and, furthermore, the anti-ulcer, anti-tumour and antiviral properties of these active compounds have been demonstrated (Arase et al 1997; Farina et al 1998; Rossi et al 2003). Recently, GA has been used in therapy as topical anti-inflammatory agent both to strengthen the skin activity of hydrocortisone through the inhibition of 11β-hydroxysteroid dehydrogenase (Teelucksingh et al 1990) and to exert a weak adrenocorticoid-like activity (Capella & Finzi 2003). Notwithstanding these interesting features, GA shows some unsuitable physicochemical properties (scarce stability, poor water solubility, etc.) that preclude its use as a therapeutic tool for the treatment of cutaneous disorders.

Numerous strategies have been studied with the aim of modifying the pharmacokinetic profile of GA and to obtain a more rational therapeutic use of this active compound (Inoue et al 1989; Salakhutdinov et al 2002; Um et al 2003; Takahashi et al 2004). Among these strategies, the synthesis of GA derivatives has been widely used (Inoue et al 1989; Um et al 2003). Unfortunately, choosing to synthesize new chemical entities from an original compound on one hand can increase the therapeutic efficacy but on the other can induce new and unexpected side-effects.

The prodrug approach represents a well-known tool widely employed to temporarily modify the physicochemical characteristics of an active compound and, consequently, increase its pharmacological activity (Kearney 1996). The prodrugs are bioreversible, pharmacologically inactive derivatives of a drug molecule that require a chemical or enzymatic transformation to release the active parent in-situ. For a successful dermal prodrug approach (Sloan 1992), the prodrug should exhibit an adequate aqueous

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stability such that its formulation in practical vehicle is possible, a controlled enzymatic conversion into the parent drug within the viable tissue and an enhanced biphasic (both lipophilic and aqueous) solubility (Guy & Hadgraft 1992). Guy & Hadgraft (1992) suggested that drug derivatization with a promoiety that possesses inherent enhancing ability would be a promising strategy to design dermal and transdermal prodrugs. This theory has been confirmed by the interesting results of our studies regarding the synthesis and the evaluation of in-vitro and in-vivo percutaneous absorption of several dermal prodrugs (Bonina et al 1995, 2001, 2002, 2003), obtained by a drug conjugation via ester linkage to different penetration enhancers. Particularly, among the pro-moieties we evaluated, polyoxyethylene possessed suitable features for a successful prodrug design (Bonina et al 2001, 2002). Polyoxyethylenes are common constituents of topical drug formulations and their skin penetration enhancing ability has been widely studied. Recently, Shin et al (2005) demonstrated that these enhancers modified the fluidity of the stratum corneum structure, favouring drug permeation through the skin.

In this paper, to assess the possibility of achieving an optimal GA dermal delivery, we investigated the in-vitro percutaneous absorption of this drug by employing the prodrug approach. With this aim, a number of polyoxyethylene esters of this drug ($\mathbf{1}_{a-e}$) have been synthesized and characterized to assess their chemical and enzymatic hydrolysis. We evaluated the ability of some of these esters (those that showed the best in-vitro profile) to inhibit methyl-nicotinate-induced skin erythema in healthy human subjects.

Materials and Methods

Materials

Melting points were determined with a Kofler hot-stage microscope (Thermovar, Reichert, Austria) and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded using a Mercury plus 400 MHz and a Mercury plus 500 MHz, respectively, equipped with VNMR software (Varian Inc., Palo Alto, CA), and using $CDCl_3$ as solvent and trimethylsilane (TMS) as internal standard. Chemical shifts values are reported in δ units (ppm) relative to TMS (1%).

The mass spectra were recorded using an API 2000 instrument equipped with a Data system software analyst 1.3 (Applied Byosystem, Foster City, USA).

Acetonitrile and water used in the HPLC procedures were of liquid-chromatography grade and were obtained from Fluka (Buchs, Switzerland). Carbopol 934 (Carbomer) was supplied by Biochim (Italy). 18-β-Glycyrrhetic acid (GA) was purchased from Sigma Chemical (Milan, Italy). Diethylene glycol, triethylene glycol, tetraethylene glycol, pentaethylene glycol, hexaethylene glycol and methyl nicotinate were purchased from Fluka. All other chemicals were of reagent grade.

General procedures for the synthesis of GA oligoethylene esters ($\mathbf{1}_{a-e}$)

In a first attempt, we tried to protect the 3-OH group in the reaction of coupling between GA (**1**) and oligoethylene glycols, but the protected derivatives were unable to react in the subsequent steps (data not shown). For this reason, we tried directly the coupling reaction without protecting the 3-OH group, using a large excess of polyoxyethylene glycol (Figure 1). The olygoethylene glycol esters ($\mathbf{1}_{a-e}$) were prepared by dissolving 2.0g (4.24 mmol) of **1** in 40 mL of a mixture composed of 15 mL of chloroform (previously distilled over CaH_2) and 25 mL of the appropriate oligoethylene glycol (**a**,**b**,**c**,**d** or **e**). Then 0.16 g (1.27 mmol) of dimethylaminopyridine (DMAP) and 0.88 g (4.24 mmol) of dicyclohexylcarbodiimide (DCC) were added. The mixture was stirred for 3 h at room temperature, then DCC was filtered off and the filtrate was added to 40 mL of an aqueous solution of KOH 0.5 M. This phase was washed twice with 20 mL of $CHCl_3$. The organic phases were collected, dried on $MgSO_4$ and evaporated to dryness in-vacuo. The products ($\mathbf{1}_{a-e}$) were purified on a silica-gel column, eluting firstly with a mixture of ethyl acetate–n-hexane (6:4), then with mixtures containing decreasing amounts of n-hexane and finally with ethyl acetate alone. The derivates $\mathbf{1}_a$ and $\mathbf{1}_b$ were white solids, and they were crystallized from n-hexane–ethyl acetate. The products $\mathbf{1}_c$, $\mathbf{1}_d$ and $\mathbf{1}_e$ were all waxes or oils and failed to crystallize. Elemental analysis was within $\pm 0.4\%$ of the theoretical values. The correct structural assignments to these products were obtained by mass spectrometry, ^{1}H and ^{13}C NMR spectra and the results are reported in Table 1. Oligoethylene glycol chemical shifts are in accordance with those reported previously (Bonina et al 2001).

Determination of chemical and enzymatic stability

To evaluate the chemical and enzymatic stability of esters $\mathbf{1}_{a-e}$, initially, 100 μL of an acetonitrilic ester solution (10^{-4} M) was diluted with 6 mL of isotonic phosphate aqueous solution (pH 7.4; $\mu = 0.5$).

Chemical stability was determined following the disappearance of ester in 3 mL of isotonic phosphate aqueous solution, thermostatted at 32°C, by HPLC analysis reported below. Enzymatic hydrolysis of esters was determined as previously reported (Bonina et al 2001). Porcine esterase was diluted 1000 fold with phosphate buffer, pH 7.4, before use. A 50-μL volume of acetonitrilic esters solution (10^{-4} M) was diluted with 3 mL of isotonic phosphate, thermostated at 37°C, and then 100 μL of esterase solution was added. The concentration of the ester in the solution was monitored by the HPLC method reported below. The formation of GA during the enzymatic hydrolysis experiment was also monitored. Plotting the decrease in prodrug and the increase in GA concentration, an exponential hydrolytic pattern was obtained. A typical trend, referred to derivative **1a**, is reported in Figure 2. Pseudo-first-order rate constants for chemical and enzymatic hydrolysis were determined from the slopes of linear plots of the logarithm of residual esters against time.

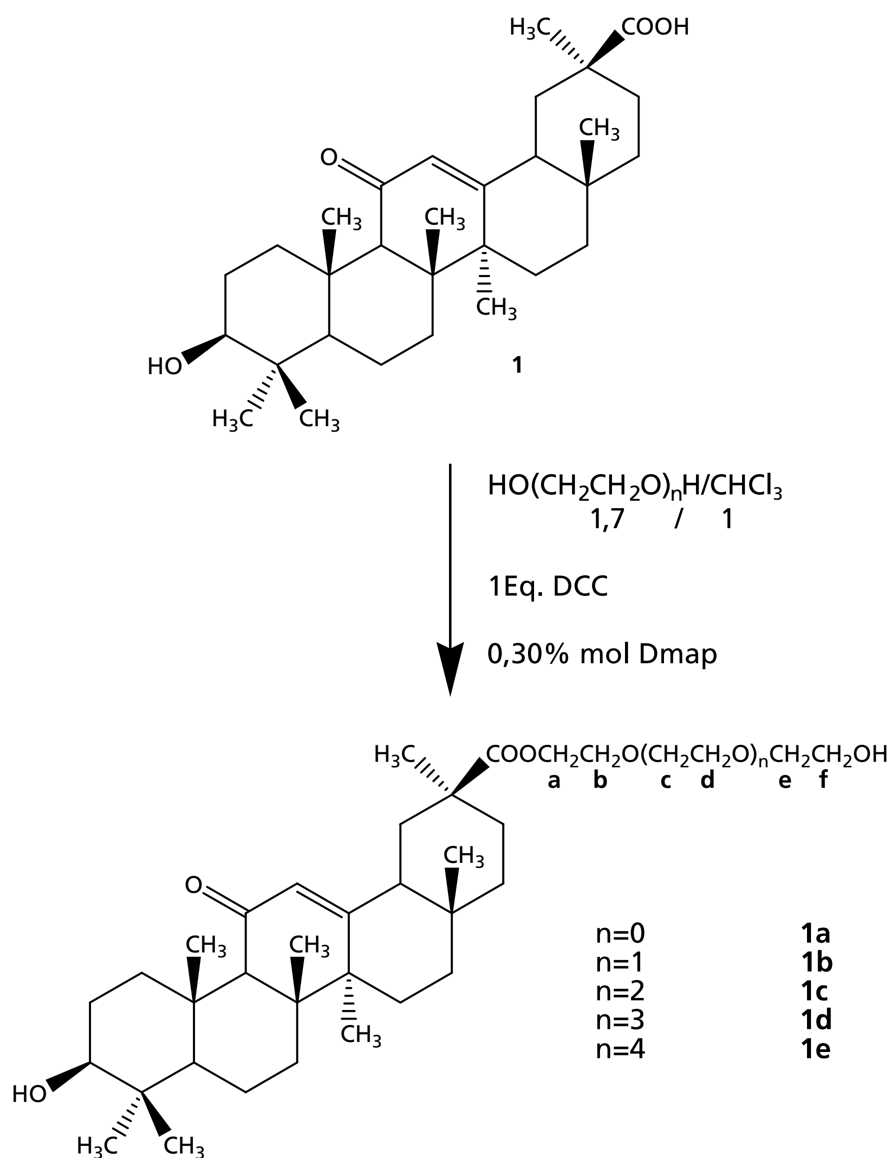

Figure 1 Synthesis of GA oligoethylene esters ($\mathbf{1}_{a\text{–}e}$).

Lipophilicity indices (log k′) and calculated partition coefficients (ClogP) of GA and esters $1_{a\text{–}e}$

Lipophilicity indices of GA and esters $\mathbf{1}_{a\text{–}e}$ were obtained by the isocratic HPLC method measuring compound retention times expressed as log k′ (Lambert 1993). It is well known that the ranking of elution in reversed-phase chromatography represents a relative scale of lipophilicity of analytes. Estimates of lipophilicity of GA and its ester derivatives were also obtained, considering the theoretically calculated values of log P (ClogP) (ACD logP DB; ChemSketch, Ontario, Canada).

In-vitro studies

For in-vitro diffusion studies, samples of adult human skin (mean age 36 ± 8 years) from breast reduction operations were processed to obtain stratum corneum/epidermis (SCE) membranes (Puglia et al 2005; Ricci et al 2005). The epidermal membranes were mounted in Franz-type diffusion cells (LGA Berkeley, CA, USA) with an exposed skin surface area of 0.75 cm^2, while the receiver compartment volume was 4.5 mL. This compartment contained a water–ethanol solution (50:50 v/v) to allow the establishment of sink conditions and to sustain permeant solubilization (Touitou & Fabin 1988). The hydro-alcoholic solution was stirred with

Table 1 Yield, melting points (m.p.), ^{1}H NMR, ^{13}C NMR, mass spectrometry (MS) and elemental analysis data for esters $\mathbf{1_{a\text{–}e}}$

Derivative	Yield	m.p.	^{1}H NMR	^{13}C NMR	MS (m/z)	Anal. (CHO)
1a	61%	>200°C	($CDCl_3$): δ 4.40–4.36 (m,1H,a), 4.17–4.13 (m,1H,a), 3.78–3.75 (m,2H,b), 3.72–3.70 (m,2H,f), 3.61 (t,3H,e)	($CDCl_3$): δ 200.41, 176.30, 168.26, 128.46, 72.15 (e), 69.35 (b), 62.40 (a), 61.64 (f)	581.0 ($M+Na^+$)	$C_{34}H_{54}O_6$
1b	57%	>200°C	($CDCl_3$): δ 4.36–4.32 (m,1H,a), 4.22–4.17 (m,1H,a), 3.74–3.64 (m,8H,b,c,d,f), 3.60 (t,2H,e)	($CDCl_3$): δ 200.41, 176,31, 169.59, 128.34, 72.44 (e), 70.48, 70.35 (c,d), 69,22 (b), 63.22 (a), 61.76 (f)	625.0 ($M+Na^+$)	$C_{36}H_{58}O_7$
1c	53%	Wax	($CDCl_3$): δ 4.33–4.31 (m,1H,a), 4.25–4.23 (m,1H,a), 3.74–3.66 (m,12H,b,c,d,f), 3.63 (t,2H,e)	($CDCl_3$): δ 200.09, 176.31, 169.24, 128.44, 72.41 (e), 70.61, 70.53 (2C), 70.32 (c,d), 69.21 (b), 63.28 (a), 61.77 (f)	646.7 (M^+), 668,5 ($M+Na^+$)	$C_{38}H_{62}O_8$
1d	49%	Oil	($CDCl_3$): δ 4.31–4.30 (m,1H,a), 4.24–4.23 (m,1H,a), 3.79–3.65 (m,16H,b,c,d,f), 3.63 (t,2H,e)	($CDCl_3$): δ 200.02, 176.27, 169.16, 128.44, 72.48 (e), 70.45, 70.39 (4C), 70.16 (c,d), 69.15 (b), 63.23 (a), 61.57 (f)	712.0 ($M+Na^+$) 728.5 ($M+K^+$)	$C_{40}H_{66}O_9$
1e	41%	Oil	($CDCl_3$): δ 4.30–4.26 (m,1H,a), 4.20–4.14 (m,1H,a), 3.79–3.61 (m,22H,b,c,d,e,f)	($CDCl_3$): δ 200.02, 176.27, 169.16, 128.44, 72.48 (e), 70.53, 70.50 (6C), 70.40 (c,d), 69.14 (b), 63.27 (a), 61.65 (f)	734.5 (M^+), 756.5 ($M+Na^+$)	$C_{42}H_{70}O_{10}$

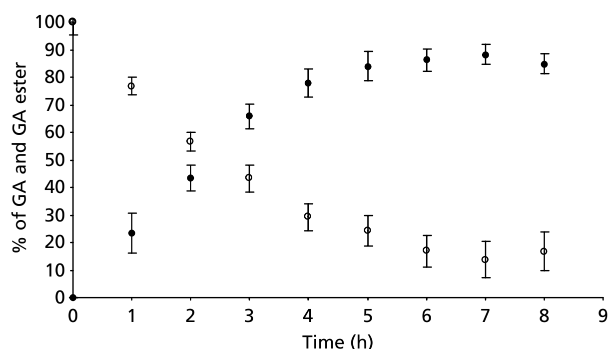

Figure 2 Time courses for GA (●) and ester **1a** (○) during enzymatic hydrolysis. Values are expressed as means ± s.d.

the help of a magnetic bar at 500 rev min^{-1} and thermostatted at 32 ± 1°C during all the experiments (Siewert et al 2003).

GA (**1**) and its esters (**1**$_{a-e}$) were dissolved in ethanol (5 mg mL^{-1}) and 200 μL was placed on the skin surface. The solvent was allowed to evaporate and the experiment was run for 36 h. Each experiment was run in duplicate using three different donors (n = 3). At intervals samples (200 μL) of receiving solution were withdrawn and replaced with fresh solution. The samples were analysed for GA (**1**) and its esters (**1**$_{a-e}$) content by HPLC as described below. Drug fluxes through the skin were calculated by plotting the cumulative amount of drug penetrating the skin against time and determining the slope of the linear portion of the curve and the *x*-intercept values (lag time) by linear regression analysis. Drug fluxes (μmol cm^{-2} h^{-1}), at steady state, were calculated by dividing the slope of the linear portion of the curve by the area of the skin surface through which diffusion took place.

HPLC analysis

The HPLC apparatus consisted of a Perkin Elmer Series 200 (45 William Street Wellsley, MA 02481-4078, USA) equipped with a Perkin Elmer photodiode array UV detector operated with Perkin Elmer's TurboChrom Navigator software.

Chromatography was performed using an ODS Hypersil column (particle size, 5 μm; 25 cm × 4.6 mm i.d; Thermohypersil, Bellefonte, PA, USA). GA (**1**) and its ester derivatives **1**$_{a-e}$ were determined by HPLC using a mobile phase consisted of water–acetonitrile (10:90). Each sample was filtered before injection with a Millex HV13 filter (Waters-Millipore Corporation, Milford, MA, USA) and a volume of 20 μL was injected into the HPLC apparatus. The flow rate was set at 1 mL min^{-1} and the effluent was continuously monitored at 254 nm. The retention times are reported in Table 2.

Chromatographic retention data are expressed as the logarithm of capacity factor (lipophilic index, log k'), defined as log k' = log [$(t_r - t_0)/t_0$], where t_r and t_0 are the retention time of the analyte and a non-retained compound (acetonitrile), respectively.

Preparation of aqueous gel

Carbomer gels, containing GA and (**1**) and its esters were prepared by dispersing carbopol 934 (Carbomer) (1.5%, w/w) in distilled water (73–75.5%, w/w) with constant stirring.

The drug (4 mmol) or its esters (4 mmol) were solubilized in ethanol (20%, w/w) together with methyl-*p*-hydroxybenzoate (0.1%, w/w). The ethanolic solution was added to the carbopol dispersion and the mixture was then neutralized and made viscous by the addition of triethanolamine (2.0%, w/w). The gels were stored at room temperature for 24 h under airtight conditions before use.

In-vivo anti-inflammatory activity of esters 1$_b$ and 1$_c$ on methyl-nicotinate-induced erythema

Instrumentation

Methyl-nicotinate-induced skin erythema was monitored by using a reflectance visible spectrophotometer X-Rite model 968 (X-Rite Inc., Grandville, MI, USA), calibrated and controlled as previously reported (Ricci et al 2005). From the reflectance spectra, obtained over the wavelength range

Table 2 Molecular mass (M_r), retention time (t_r), chemical and enzymatic hydrolysis (t½) and calculated partition coefficient (Clog P) of GA and its esters (**1**$_{a-e}$)

Compound	M_r	t½ (h)		ClogP	t_r
		Buffer pH 7.4	Porcine esterase		
GA	470.7	—	—	6.57 ± 0.4	5.21
1$_a$	558.7	533.2 ± 96.5	2.9 ± 0.3	6.29 ± 0.5	4.95
1$_b$	602.8	693.1 ± 88.6	6.1 ± 0.5	5.93 ± 0.5	4.88
1$_c$	646.8	630.1 ± 91.6	7.3 ± 0.4	5.58 ± 0.6	4.75
1$_d$	690.9	528.9 ± 79.6	18.3 ± 0.7	5.22 ± 0.7	4.64
1$_e$	735.0	628.4 ± 83.6	19.8 ± 0.6	4.86 ± 0.7	4.62

400–700 nm, the erythema index (E.I.) was calculated using Equation 1:

$$E.I. = 100[\log 1/R_{560} + 1.5(\log 1/R_{540} + \log 1/R_{580}) - 2(\log 1/R_{510} + \log 1/R_{610})] \quad (1)$$

where 1/R is the inverse reflectance at a specific wavelength (560, 540, 580, 510 or 610).

Protocol

In-vivo experiments were performed on ten subjects of both sexes (age range 25–35 years). They were recruited after medical screening, including filling in a health questionnaire followed by physical examination of the application sites. After they were fully informed of the nature of the study and of the procedures involved they gave their written consent. The participants did not suffer from any ailment and were not on any medication at the time of the study. They were rested for 15 min before the experiments and room conditions were set at 22 ± 2°C and 40–50% relative humidity.

For each subject, ten sites on the ventral surface of each forearm were defined using a circular template (1 cm^2) and demarcated with permanent ink.

For each subject, one of the ten sites on each forearm was used as a control, by applying 50 mg gel without active compounds, and the other nine sites were treated with 50 mg of gel containing GA or esters $\mathbf{1_b}$ and $\mathbf{1_c}$. The preparations were spread uniformly on the site by means of a solid glass rod. The sites were then occluded for 3 h using Hill Top Chambers (Hill Top Research, Cincinnati, OH, USA). After the occlusion period, the chambers were removed and the skin surface was washed to remove the gel and allowed to dry for 15 min. On each pre-treated site, methyl nicotinate aqueous solution (0.5%, w/v) was applied at different times after gel removal: immediately (t = 0), 3 and 6 h later (t = 3 and t = 6, respectively). Methyl nicotinate was applied on the skin surface for 1 min using a Hill Top Chamber (1 cm^2), the cotton pad of which was saturated with 200 μL of methyl nicotinate solution, and the induced erythema was monitored for 100 min. E.I. baseline values were taken at each designated site before application of gel formulation and they were subtracted from the E.I. values obtained after methyl nicotinate application at each time point to obtain ΔE.I. values. For each site, the area under the response (ΔE.I)–time curve (AUC) was computed using the trapezoidal rule.

To better outline the results obtained, from AUC values the percentage of inhibition of the erythema (P.I.E.) was calculated using Equation 2:

$$\text{Inhibition (\%)} = [(AUC_{(C)} - AUC_{(T)})/AUC_{(C)}] \times 100 \quad (2)$$

where $AUC_{(C)}$ is the area under the response–time curve of the vehicle–treated site (control) and $AUC_{(T)}$ is the area under the response–time curve of the drug-treated site.

Statistical analysis

Statistical differences were determined using repeated measure analysis of variance followed by the Bonferroni–Dunn post-hoc pair-wise comparison procedure. $P < 0.05$ was considered significant in this study.

Results and Discussion

Chemical and enzymatic stability

In Table 2 the chemical and enzymatic stability data of esters $\mathbf{1_{a–e}}$ are reported. The results showed that most of the synthesized esters had a good stability in phosphate buffer at pH 7.4 and were hydrolysed by porcine esterase. Regarding the chemical stability of esters $\mathbf{1_{a–e}}$, there was no significant difference in their hydrolysis rates as the length of the polyoxyethylene chain increased ($P > 0.05$). A different trend was observed in the enzymatic stability studies, in which ester derivatives $\mathbf{1_{a–c}}$ were more readily hydrolysed by porcine esterase than esters $\mathbf{1_{d–e}}$, which showed t½ values above 12 h.

It is important to note that $\mathbf{1_b}$ and $\mathbf{1_c}$ showed a similar hydrolysis rate ($P > 0.05$) and that no significant differences were observed also comparing enzymatic hydrolysis data of esters $\mathbf{1_d}$ and $\mathbf{1_e}$ t ($P > 0.05$).

Physicochemical characteristics of esters $1_{a–e}$

Physicochemical parameters, such as aqueous solubility and lipophilicity, influence the membrane flux, therapeutic activity and pharmacokinetic profiles of medicines (Monene et al 2005). To assess the lipophilicity of esters $\mathbf{1_{a–e}}$ two different parameters were considered: CLOG data from theoretical calculation and log k′ chromatographic indices. GA had a very high CLOGP value (>6) and generally, for this kind of substance, the rate of transfer between the stratum corneum and the epidermis will be slow and will limit absorption across the skin. The esterification of the carboxylic group of GA gave esters $\mathbf{1_{a–e}}$ with decreased lipophilicity compared with the parent drug (Table 2), and as the polyoxyethylene chain grew longer this parameter decreased as a result of the increasing hydrophilicity introduced in these molecules by adding new polyoxyethylene units. In fact the log P values obtained for the synthesized esters were lower than the value obtained for GA, even though they were not very close to the optimal range (2–4) recommended to favour dermal absorption (Magnusson et al 2004a).

With regard to the log k′ values, this determination failed since no significant differences were observed among the synthesized esters.

Water solubility is regarded as an important parameter for a successful dermal prodrug design and it is widely recognized (Sloan & Wasdo 2003) that a dermal prodrug should possess an increased aqueous solubility compared with the parent drug and an adequate lipophilicity. For the synthesized esters ($\mathbf{1_{a–e}}$), we have not been able to determine experimentally their water solubility, and also the application of Yalkowsky theoretical methods (Yalkowsky & Valvani 1980; Yalkowsky et al 1983) to estimate the water solubility failed, producing unrealistic and unreproducible data.

In-vitro permeation experiments

Figure 3 shows a plot of the cumulative amount of GA and its derivatives permeated through human SCE membranes as a function of time. In the case of esters $\mathbf{1_{a–e}}$ skin permeation experiments, intact ester was found in the receptor phase together with a variable amount of GA (2–5%) compared with the amount of ester permeated. The observed hydrolysis should be ascribed mainly to the chemical hydrolysis given that in previous in-vitro skin permeation studies of dermal prodrugs (Bonina et al 2001), we observed a very poor enzymatic activity of SCE membranes. Furthermore, the large amount of ethanol used in the receptor compartment for ensuring the sink condition could notably decrease the chemical hydrolysis in this medium with respect to that obtained in pH 7.4 buffer aqueous solution used for in-vitro chemical stability studies.

GA flux values (J_{SS}) calculated from the linear segments at steady state are reported in Figure 4. As shown, the application of esters $\mathbf{1_b}$ and $\mathbf{1_c}$ onto the skin resulted in a higher permeation rate compared with GA, while there were no significant differences among GA and esters $\mathbf{1_a}$, $\mathbf{1_d}$ and $\mathbf{1_e}$ permeation profiles ($P > 0.05$). The GA skin permeation enhancement, shown by esters $\mathbf{1_b}$ and $\mathbf{1_c}$, could be due to an optimal balance between lipophilicity and hydrophilicity values and, probably, can be explained on the basis of their increased water solubility with respect to parent drug. As regards ester $\mathbf{1_d}$ and $\mathbf{1_e}$, both their elevated molecular size (Magnusson et al 2004b) and their ability to form H-bonds (due to more polyoxyethylene groups) (Lien & Gao 1995) influenced their diffusional properties, reducing their permeation rate through the skin.

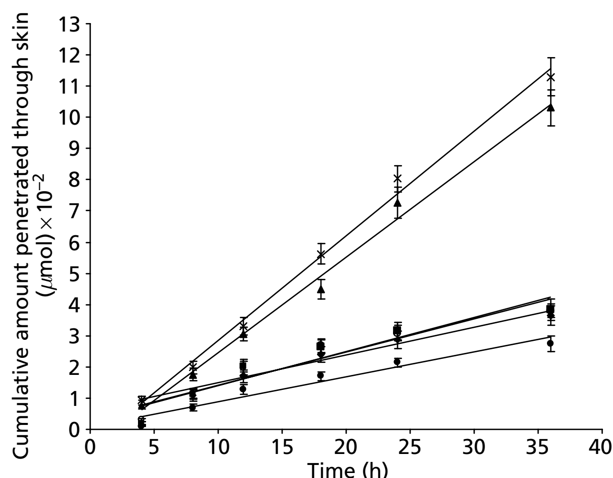

Figure 3 Plot of cumulative amount (μmol) of GA (◆) and ester $\mathbf{1_a}$ (■), $\mathbf{1_b}$ (▲), $\mathbf{1_c}$ (×), $\mathbf{1_d}$ (○) and $\mathbf{1_e}$ (●) penetrated through excised human skin versus time. Values are expressed as means ± s.d., n = 10.

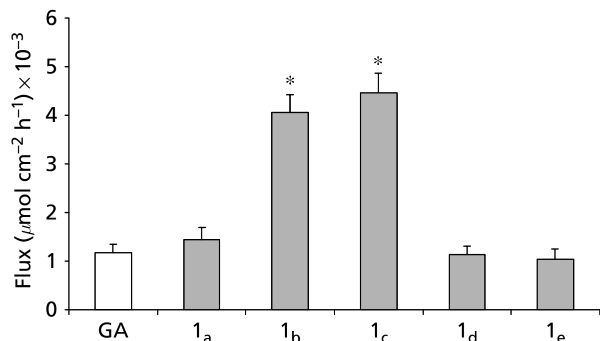

Figure 4 Drug steady-state fluxes through excised human skin for GA and its esters ($\mathbf{1_{a–e}}$). Values are expressed as means ± s.d., n = 10. *$P <$ 0.05, GA vs $\mathbf{1_b}$, $\mathbf{1_c}$.

In-vivo anti-inflammatory activity

Since the results obtained in-vitro were very promising, we thought it was worthwhile investigating the topical anti-inflammatory activity of esters $\mathbf{1_b}$ and $\mathbf{1_c}$, which had shown the best in-vitro results, in healthy subjects. With this aim, carbomer gels containing esters $\mathbf{1_b}$ and $\mathbf{1_c}$ (4 mmol/100 g) or GA (4 mmol/100 g), were formulated.

From the ΔE.I. values, calculated at each site and at different times, it was possible to monitor the extent of methyl-nicotinate-induced skin erythema and the ability of esters $\mathbf{1_b}$ and $\mathbf{1_c}$ to inhibit this process after their preventive application onto the skin, using carbopol gels as vehicle formulations. Plotting ΔE.I. values versus time, AUC values were determined for each subject by calculating the areas between the response curve and the *x*-axis, and the mean AUC values are reported in Table 3. AUC values were inversely related to the tested substances' ability to inhibit methyl-nicotinate-induced erythema. Methyl nicotinate application was effected at different times (t = 0 h, t = 3 h, t = 6 h) after active compound removal.

As reported in Table 3, at t = 0 GA was more effective than the esters $\mathbf{1_b}$ or $\mathbf{1_c}$ in inhibiting induced erythema, while its anti-erythematous efficacy noticeably decreased at t = 3 h and it was about 10% 6 h (t = 6) after gel removal.

The P.I.E. value (Figure 5) obtained with GA was maximal when methyl nicotinate was applied immediately after gel removal (t = 0), while it notably decreased at t = 6 h. Since it is reasonable to suppose that the anti-inflammatory activity is related to drug concentration in viable epidermis, these results suggest a rapid depletion of GA in the viable tissue.

A different trend was observed with esters $\mathbf{1_b}$ and $\mathbf{1_c}$. The former showed the highest anti-inflammatory activity at t = 6 h and a very low P.I.E. value at t = 3 h (about 20%), while the latter showed very similar values of P.I.E. at t = 3 and t = 6 h. The anti-inflammatory activity profiles, obtained in particular for $\mathbf{1_b}$ and $\mathbf{1_c}$, could be due to either a different hydrolysis rate of the prodrugs within the skin or retention in the stratum corneum where a reservoir may be established. The last hypothesis could justify the sustained effect, obtained particularly for $\mathbf{1_b}$, which, as reported by others (El-Kattan et al 2001), is strictly related to the lipophilic character of the substance applied onto the skin.

Conclusion

The results obtained in this study showed that the use of polyoxyethylene glycols appeared suitable to obtain GA dermal prodrugs endowed with chemical stability, enzymatic lability

Table 3 AUC_{0-100} values obtained by pretreating human skin sites with gel formulations containing GA and esters $\mathbf{1_b}$ and $\mathbf{1_c}$ and applying methyl nicotinate solution immediately (t = 0), and at 3 h (t = 3) or 6 h (t = 6) after their removal

Subjects	t = 0			t = 3			t = 6			Control
	GA	$\mathbf{1_b}$	$\mathbf{1_c}$	GA	$\mathbf{1_b}$	$\mathbf{1_c}$	GA	$\mathbf{1_b}$	$\mathbf{1_c}$	
A	669.5	934.8	831.1	754.5	854.6	751.2	936.0	414.5	810.5	1091.2
B	679.5	930.1	921.4	874.9	718.8	613.4	1015.1	512.9	590.9	1132.8
C	762.2	750.4	909.4	859.9	1015.9	829.7	1136.7	606.2	667.2	1321.5
D	752.1	862.0	824.9	893.6	890.6	800.5	991.1	698.3	680.3	1221.4
E	643.5	1206.4	726.9	783.6	754.9	696.4	846.2	530.6	670.1	988.6
F	503.8	749.6	729.7	881.7	913.8	680.6	1143.6	503.9	821.7	1022.9
G	648.4	915.5	873.9	850.6	878.3	618.5	1011.4	644.4	742.1	1029.7
H	718.1	924.2	847.8	918.1	791.6	626.3	991.4	581.1	749.3	1121.3
I	701.3	918.3	821.1	884.3	719.4	619.7	1018.2	576.3	629.1	1214.6
L	668.6	897.6	816.7	821.6	884.3	674.2	928.3	619.2	617.3	1199.2
Mean	674.7	908.9	830.3	852.3	842.2	691.0	1001.8	568.7	697.9	1134.3

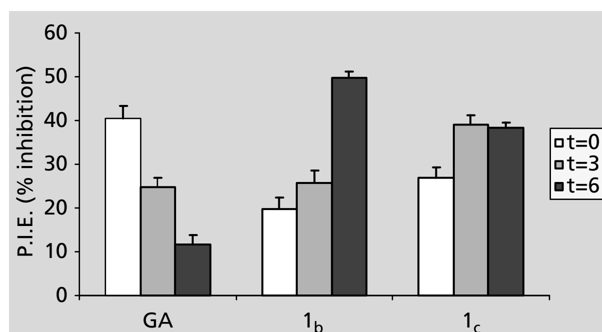

Figure 5 Percentage of inhibition of methyl-nicotinate-induced erythema (P.I.E.) for GA and $\mathbf{1_b}$ and $\mathbf{1_c}$ ester derivatives. Data represent mean ± s.d., n = 10.

and increased in-vitro skin permeation. Particularly, the application of esters $\mathbf{1_b}$ and $\mathbf{1_c}$ onto the skin resulted in a higher permeation rate compared with GA. Furthermore, we observed an appreciable and sustained in-vivo topical anti-inflammatory activity of esters $\mathbf{1_b}$ and $\mathbf{1_c}$ compared with the parent drug.

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