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ORIGINAL ARTICLE

INFLUENCE OF VEHICLES AND PENETRATION ENHANCERS ON ANTI-INFLAMMATORY EFFECT OF 18-β GLYCYRRHETINIC ACID: KINETIC MODELLING OF DRUG RELEASE, *IN VIVO* AND *EX VIVO* EXPERIMENTS

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Abstract

Topical formulations of 18-β glycyrrhetinic acid (18-β GA) were designed for use in relieving inflammatory and painful conditions of the skin. Formulations were containing penetration enhancers that differ in penetration enhancing mechanisms. Anti-inflammatory effects of formulations and effects of penetration enhancers on penetration and permeation of the drug through rat skin were investigated. The total amount of 18-β GA permeated from the base oil/water emulsion (53.19 ± 22.25 mcg/cm^2) was approximately twice higher than the base oleaginous cream (29.17 ± 3.85 mcg/cm^2) while there was no 18-β GA permeation from the base hydrogel formulation to the skin ($p < 0.05$). Incorporation of propylene glycol was generally found to increase 18-β GA permeation to the skin. The highest oedema inhibiting activity was achieved in the oil/water emulsion containing propylene glycol followed by the base oil/water emulsion without a penetration enhancer ($p < 0.05$). This result was consistent with the *ex vivo* study. Limonene and oleic acid were found to be insufficient in 18-β GA permeation to the skin.

Rezumat

Au fost elaborate formulări topice ale acidului gliciretinic 18-β (GA 18-β), cu promotori de penetrare, pentru utilizarea acestora în ameliorarea bolilor inflamatorii ale pielii. Au fost cercetate efectele antiinflamatoare ale formulărilor și efectele promotorilor de penetrare asupra penetrării și permeării medicamentului prin pielea șobolanului. Cantitatea totală de 18-β GA pătrunsă în piele din emulsia de bază U/A (53,19 ± 22,25 mcg/cm^2) a fost aproximativ de două ori mai mare decât din crema oleaginoasă (29,17 ± 3,85 mcg/cm^2). Nu a existat o permeare de 18-β GA din formularea hidrogelului de bază ($p < 0,05$). Încorporarea propilenglicolului a crescut permeabilitatea GA-18-β în piele. Cea mai mare activitate de inhibare a edemelor a fost obținută cu emulsia U/A conținând propilenglicol, urmată de emulsia U/A de bază fără promotor de penetrare ($p < 0,05$). Acest rezultat a fost în concordanță cu studiul *ex vivo*. Limonenul și acidul oleic s-au dovedit a fi insuficiente în creșterea permeabilității GA-18-β prin piele.

Keywords: 18-β glycyrrhetinic acid, enoxolone, anti-inflammatory drugs, skin penetration, penetration enhancers

Introduction

18-β glycyrrhetinic acid (enoloxolone) (18-β GA), one of pentacyclic triterpenes existing in the root of *Glycyrrhiza glabra* L., is available in skin products to remove inflammation, acne and allergy [1, 2]. Anti-inflammatory properties of 18-β GA relate to the inhibitory effect on the production of superoxide radicals by neutrophils, primary mediators of inflammation. It was reported that it provided anti-inflammatory effects by attenuating the formation of excessive nitric oxide, prostaglandin E2 and intracellular reactive oxygen species and by suppressing the expression of pro-inflammatory genes through the inhibition of NF-κB and phosphoinositide-3-kinase activity [3]. Its anti-tumoural activity was reported [4]. Furthermore, it has antibacterial and antifungal properties due to its steroid-like structure [5]. 18-β GA is used in cosmeceuticals for its firming, moisturizing, whitening and antiaging effects to protect skin health and condition

the skin. Its skin lightening effect is attributed to the inhibition of tyrosinase activity in melanocytes [6].

Penetration enhancers are often incorporated in topical products to achieve effective treatment *via* the transdermal route. They are sorption promoters that increase transdermal drug delivery by reducing skin barrier resistance [7, 8]. Glycols, terpenes and fatty acids are commonly used as penetration enhancers. They display different mechanisms through upper layers of the skin, mainly the *stratum corneum* [9]. They are able to promote delivery of both lipophilic and hydrophilic actives through the skin. Oleic acid (OA) is the most popular long chain fatty acid that enhances percutaneous drug absorption by disrupting the skin's intercellular lipid packing. A polar head group attached to its alkyl chain conducts its potential enhancement function. Whereas, propylene glycol (PG) causes insignificant disruption of intercellular lipid packing within *stratum corneum* bilayers. PG first penetrates through tissues itself and then alters the thermodynamic activity of the drug in a vehicle. Subsequently, the drug diffuses through the *stratum corneum* by modification of driving forces for diffusion. Limonene (L) is known as one of the most effective terpene enhancers. L displays a greater difference than many types of terpenes probably because it displays multiple mechanisms. L provides partial extraction of *stratum corneum* lipids and phase separation within *stratum corneum* lipid lamellae while increasing drug solubility in the skin [10, 11].

In this study, effects of various enhancers on penetration of 18-β GA incorporated in traditional vehicles (hydrogel (G), oil/water (o/w) emulsion (E) and oleaginous cream (OC)) were investigated for relieving inflammatory conditions of the skin. Physical characterization experiments were performed on topical formulations. Earlier studies demonstrated that *in vivo* and *ex vivo* tests on rats can be used to investigate characteristics required for skin delivery from an active or a vehicle [12, 13]. Thus, effects of these penetration enhancers on anti-inflammatory effectiveness of 18-β GA were determined by hindpaw oedema test on Wistar albino rats. In the next step, penetration and permeation of the active through the skin was investigated in an *ex vivo* study.

Materials and Methods

Materials

18-β glycyrrhetinic acid (18-β GA) was kindly provided from Nobel İlaç San. ve Tic. A.Ş., Turkey. Propylene glycol (PG), oleic acid (OA), DL-limonene (L) and Tween® 80 were purchased from Merck (Germany). Cetyl alcohol and liquid paraffin were purchased from Sigma-Aldrich (Germany). Xanthan gum was purchased from Inner Mongolia Jianlong Biochemical Co., Ltd. (China). All other reagents and chemicals were of analytical grade.

Topical formulations

Compositions of base 18-β GA formulations (G, E and OA) are presented in Table I. Base hydrogel (G), o/w emulsion (E) and oleaginous cream (OC) formulations were prepared. The base G formulation containing xanthan gum in water was prepared by keeping the mixture for 24 h. Then, 18-β GA was added to the hydrogel by stirring using a HS-100D propeller mixer (Daihan Scientific, Korea) at 400 rpm for 1 min. The mixture was additionally kept in an ultrasonic bath (Bersonic, Turkey) for 1 min. The base E formulation was prepared by addition of a hot aqueous Tween® 80 solution (7.7%, w/w) to the lipophilic phase containing 18-β GA, vaseline, liquid paraffin and cetyl alcohol by stirring at 70ºC and 400 rpm for 2 minutes. This emulsion was continued to be stirred until it cooled to the room temperature. The base OC formulation was prepared with addition of 18-β GA to the hot vaseline and liquid paraffin mixture by stirring at 70ºC for 2 min. and the mixture was cooled down to the room temperature. Methylparaben and propylparaben were incorporated into aqueous and lipophilic phases of formulations, respectively.

Table I

Constituents of base formulations

Constituents (%)	G	E	OC
18-β GA	3	3	3
Xanthan gum	2	-	-
Vaseline	-	5	85
Liquid paraffine	-	17	12
Cetyl alcohol	-	5	-
Tween® 80	-	5	-
Distilled water	95	65	-
Methylparaben	0.2	0.2	-
Propylparaben	-	0.2	0.2

G, gel; E, o/w emulsion; OC, oleaginous cream

Formulations containing penetration enhancers (PG, OA and L) were prepared under the same conditions (Table II) [14].

Table II

Formulations containing penetration enhancers

Formulations	Enhancers (%)		
	PG	L	OA
G*	-	-	-
G-PG	10	-	-
G-L	-	1	-
E*	-	-	-
E-PG	10	-	-
E-OA	-	-	5
E-L	-	3	-
OC*	-	-	-
OC-PG	10	-	-
OC-OA	-	-	5

*G, E and OC are base formulations. PG, propylene glycol; L, limonene; OA, oleic acid

Quantification of 18-β GA

UV spectroscopy and HPLC methods for quantification of 18-β GA in samples obtained during experiments were validated according to the ICH guideline [15]. Thus, both of UV spectroscopy and HPLC methods were confirmed to be fit for their intended purpose during the research. Linearity, intra-day and inter-day precision, accuracy, recovery and specifity were determined to verify methods. Each verification analysis was repeated 6 times.

UV spectroscopy analysis. Analytical quantification by UV spectroscopy (Shimadzu Spectrophotometer UV-1700, Japan) was performed to investigate the solubility of 18-β GA and *in vitro* drug release properties of formulations in the receptor phase. pH 7.4 phosphate buffer solution:ethanol:propylene glycol (PBS:EtOH:PG) (2:1:1) mixture was used as the receptor phase. This method was preferred, since it's a rapid, cost-efficient and reproducible technique with proper detection limits for such experiments. For this purpose, a calibration curve was drawn using concentrations of standard 18-β GA solutions (2, 4, 6, 8, 10 and 12 µg/mL) against absorbance values obtained at 256 nm.

HPLC analysis. Analytical quantification by HPLC was performed to investigate *ex vivo* skin penetration characteristics of 18-β GA. A HPLC apparatus (Shimadzu LC-20AT) was equipped with an UV detector (Shimadzu SPD-20A-UV), an autosampler (Shimadzu SIL-20A) and a column oven (Shimadzu CTO-10AS) kept at 40ºC. A GL Sciences Inertsil C18 column (150 mm x 4.6) was also equipped to the apparatus for detection. Samples were detected under the 1 mL/min flow rate of the acetonitrile:0.02 PBS (1:1, v/v) mixture as the mobile phase at 248 nm.

A stock standard solution of 18-β GA was prepared in the receptor phase at a concentration of 0.1 mg/mL. A working standard solution of 18-β GA was also prepared at the concentration of 10 µg/mL by using the same solvent. To evaluate linearity of the method under the selected conditions, drug determination was carried out at six concentrations (6.25 - 500 ng/mL) in the mobile phase for the calibration curve.

Solubility of 18-β GA in the receptor phase

The solubility of 18-β GA was determined in the receptor phase according to the method reported in USP XIX [14, 16]. 10 mL of a pH 7.4 PBS:EtOH:PG (2:1:1, v/v/v) mixture as the receptor phase were placed in four 25 mL flasks for this purpose. A quantity of 18-β GA was placed in each flask which was greater than the quantity expected to dissolve in the receptor phase. Flasks were tightly closed and they were placed in a constant temperature water bath (Daihan Scientific, Korea) at 32 ± 0.5ºC. The apparatus was maintained under a continuous agitation at 160 rpm for 24 h. Dispersions were then filtered through S & S^{5893} blue ribbon papers (2 µm pore size, Schleicher & Schuell, Germany). Measured portions of clear supernatants were removed and properly diluted. The solubility of 18-β GA in the receptor phase was determined spectrophotometrically at 256 nm.

In vitro drug release from formulations

0.45 µm cellulose acetate membranes (Sartorius, Germany) were used between two halves of Franz-type diffusion cells with 3.15 cm^2 surface area and 33.2 mL volume [17]. Receptor chamber of diffusion cells were filled with the receptor phase. 0.5 gram of topical formulation was placed in the donor phase on to the membrane. The receptor phase was kept at a constant temperature of 32 ± 0.5ºC during this study. Samples were taken from the receptor phase at certain time intervals. Cumulative amounts of released 18-β GA were determined by UV spectroscopy at 256 nm after proper dilution of samples. They were plotted as a function of time. Drug release profiles were evaluated by using different kinetic models (zero order, first order and Higuchi square-root model) [18, 19]. "n" values of Korsmeyer-Peppas kinetic model were considered to specify drug release mechanisms, especially when drug release comprises more than one type of release mechanisms.

In vivo assessment of anti-inflammatory effects of topical formulations

For this purpose, Wistar Albino rats (150 - 200 g) were provided from "Aziz Sancar" Institute of Experimental Medicine (formerly known as DETAE, Istanbul University Institute for Experimental Medicine). The *in vivo* experimental protocol was approved by the Local Ethical Committee of Animal Experiments at the same institute (06.03.2014, No. 2014/30). Animals were housed in plastic cages at a constant temperature (22 ± 1ºC) and humidity (60 ± 1%) under 12 h light-dark cycles. They were given the standard laboratory diet and tap water *ad libitum*. Rats were acclimated to the laboratory at least 7 days prior to experiments. Animals were divided into 12 groups of 3 animals (Table III). Hindpaws of rats were measured with a plethysmometer (Ugo Basile, Italy) and paw volumes (mL) were obtained. While right hindpaws of animals were used for control, oedema was provided on their left hindpaws. The first group was the blank group treated with 0.25% (w/v) CMC-Na aqueous solution orally. 18-β GA was suspended in 0.25 % CMC-Na aqueous solution (0.3%, w/v) for application to the oral group (18 mg/kg). Oedema in hindpaws of rats was provided using CMC-Na. Inflammation was induced by intraplantar injection of 0.1 mL of 1% (w/v) CMC-Na solution in saline in the left hindpaw of all the rats 30 and 60 min later oral and topical applications. 0.2 g formulation containing 6 mg 18-β GA was applied on 10 groups of rats for each topical formulation. Formulations were applied to the planter surface of the left hind paw by gently rubbing 50 times with the index finger. Intensity of oedema was assessed by measuring paw volumes with the

blethysmometer. Measurements were carried out at predetermined time intervals for 6 hours. Swelling % and oedema inhibition % were calculated by using Equations 1 and 2 [20, 21]:

$$\text{Swelling (\%)} = [(V_t - V_0)/V_0] * 100, \quad (1)$$

$$\text{Oedema inhibition (\%)} = \{[(V_t - V_0)_{control} - (V_t - V_0)_{application}]/(V_t - V_0)_{control}\} * 100, \quad (2)$$

where V_0 is the mean volume of hindpaws before application and V_t is the mean volume after drug application by oral and transdermal routes at time (t).

Table III

Animal groups for determination of anti-inflammatory effects of formulations

Groups	Application
Blank	Oral 0.25% CMC-Na aqueous solution
Oral susp.	Oral 18-β GA aqueous suspension (20 mg/kg)
G	Topical application
G-PG	
G-L	
E	
E-PG	
E-OA	
E-L	
OC	
OC-PG	
OC-OA	

G, E and OC are base formulations. PG, propylene glycol; L, limonene; OA, oleic acid

Ex vivo skin penetration and permeation studies

Franz-type diffusion cells were used to determine 18-β GA permeation through the skin [22]. For *ex vivo* skin penetration and permeation assessments, abdomens of animals were precisely shaved 2 days prior to the *in vivo* study. Shaved full-thickness abdominal skins of rats were taken after they were sacrificed following the *in vivo* assessment. The underlying fatty tissue was removed by blunt dissection without damaging the epidermal surface. They were placed between two halves of cells. 0.5 g formulations were applied on the top of the skin placed on the donor chamber of cells. The same solution, a pH 7.4 PBS: EtOH:PG (2:1:1) mixture, was used as the receptor phase. This study was continued at 37 ± 1ºC constant temperature for 48 hours [23, 24]. Amount of permeated 18-β GA was assayed in samples collected from the receptor phase by HPLC at predetermined time intervals. Three replicates were conducted for each formulation under the sink condition.

The cumulative amount (Q_n, mcg/cm^2) of 18-β GA permeated through the skin was calculated using Equation 3. They were plotted as the function of time (t, h) [22, 25].

$$Q_n = \frac{C_n V_0 + \sum_{i=1}^{n-1} C_i V_i}{A}, \quad (3)$$

where C_n is the 18-β GA concentration in the receptor phase at the nth sampling interval. A is the effective diffusion area (surface of the sample cell), V_0 and V_i are volumes of the receptor phase in the individual Franz cell and the sample, respectively. $\sum_{i=1}^{n-1} C_i$ is the sum of 18-β GA concentration determined at sampling intervals 1 through n-1.

The steady state flux of 18-β GA (J_s, mcg/cm^2/h) was determined from the slope of the linear part of the plot using the linear regression analysis ($r > 0.99$). It was calculated using Equation 4.

$$J_s = C_0 \frac{KD}{L} = C_0 K_p, \quad (4)$$

where C_0 is the constant drug concentration in the donor phase, D is the diffusion coefficient, L is the thickness of the membrane, K is the partition coefficient of the drug and the vehicle, and K_p is the permeability coefficient.

Skin penetration of 18-β GA was investigated recovering the same abdominal rat skins placed on the receptor chamber of Franz-type diffusion cells. Excess formulation in contact with the *stratum corneum* was carefully removed using cotton buds from the diffusion area [26]. Circular PVC adhesive tape pieces (Ve-ge®, İzmir, Turkey) were provided in approx. 1 cm semi-diameter. They were applied over the diffusion area. Subsequently, a light pressure was applied and they were removed with a forceps. The first two strips were discarded since they were containing the formulation lodged within crevices of the skin surface rather than being deposited within the tissue. The next 10 adhesive tape pieces were then applied to the same area using a uniform firm pressure and removed with uniform force rapidly. They were collected in a 25 mL flask for extracting 18-β GA content. For this purpose, 10 mL ethanol was added to each flask for each formulation and all flasks were tightly closed. They were fixed in the constant temperature water bath at 25 ± 1ºC. The apparatus was maintained at 160 rpm continuous agitation for 24 h. Dispersions were then filtered through S & S^{5893} blue ribbon papers (2 µm pore size, Schleicher & Schuell, Germany). A measured portion of clear supernatants was removed and their 18-β GA content was determined by HPLC.

Data treatment and statistics

Drug release and permeation profiles of formulations obtained from *in vitro* and *ex vivo* experiments and data obtained from tape stripping and *in vivo* experiments were statistically compared with one-way analysis of variance (ANOVA) test and the subsequent Fisher post hoc pairwise least significant difference test (PLSD). For this purpose, the Minitab® 18 Statistical Software was employed. The significance level was set as $\alpha = 0.05$.

Results and Discussion

Analytical quantification

The purpose of validation is to show that processes involved in the development of a dosage form, production and analytical testing can be performed in an effective and reproducible manner. It was confirmed that both of the analytical methods were fit for their intended purpose during the research according to instructions of the ICH Harmonised Tripartite Guideline [15]. The UV absorption spectra of 18-β GA showed λ_{max} at 256 nm in the pH 7.4 PBS:EtOH:PG (2:1:1) mixture (Figure 1a). The representative linear equation was A = aC + b, where A is the absorbance, a is the slope, C is the concentration and b is the intercept. The regression equation was A = 38.94C + 0.267 (correlation coefficient, r = 0.9991). Additionally, the representative linear equation was determined as A = 22.41C + 1281.3 (r = 0.9963) for HPLC analysis (Figure 1b). The retention time of 18-β GA was obtained as 4.5 min. Limits of detection (LOD) and quantification (LOQ) were 0.24 ng/mL and 0.74 ng/mL, respectively. Relative standard deviations for accuracy, intra-day and inter-day precision of the methods were below 2%. Recovery of 18-β GA was found to be between 100.64 ± 0.42% and 101.37 ± 0.52%.

a **b**

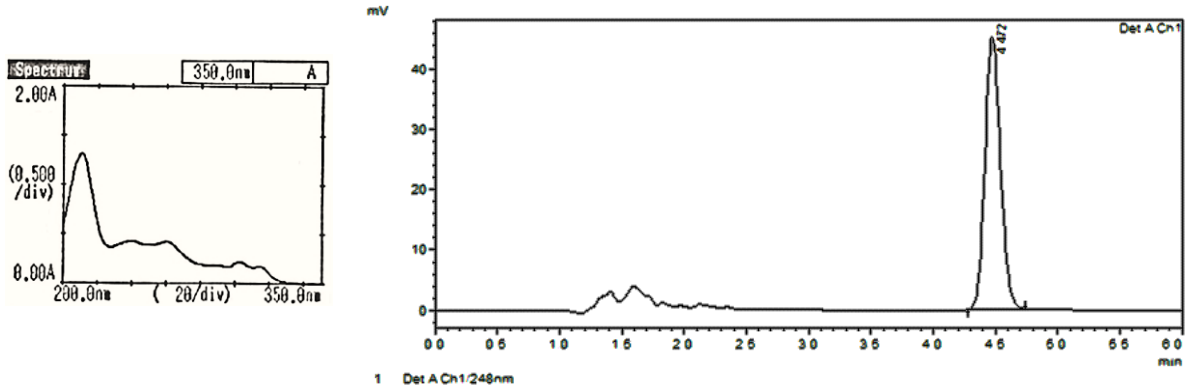

Figure 1.
The absorption spectra of 18-β GA (6 μg/mL) in the pH 7.4 PBS:EtOH:PG (2:1:1) mixture (**a**) and the HPLC graph of 18-β GA (10 μg/mL) in the acetonitrile:0.02 PBS (1:1) mixture (**b**)

Solubility of 18-β GA in the receptor phase

The solubility of 18-β GA in the pH 7.4 PBS:EtOH:PG (2:1:1) mixture as the receptor phase was found to be 7.058 ± 0.67 mg/mL at 32 ± 0.5ºC. After trying different solvent mixtures, it was concluded that it would be appropriate to use this mixture as a receptor phase. In the case of lipophilic substances such as 18-β GA, it is difficult to provide the sink condition. To achieve the sink condition, the receptor medium must have a relatively high capacity to solve or carry away the drug and the receptor media should not exceed 10% of drug solubility in the releasing matrix at the end of the test. Composition of the receptor phase can provide the suitable sink condition. The sink condition can be achieved by the use of alcohols or glycols in the receptor phase. For this purpose, EtOH and PG are often added to pH 7.4 PBS up to 1:1 [27-30].

In vitro 18-β GA release from the formulations

In *vitro* release study cannot be certainly regarded as a criterion for drug permeation through the skin. However, it may help someone to estimate some reasons of low drug penetration rate when penetration enhancing mechanism of penetration enhancers does not rule [31]. In a comparison of vehicles, the formulation G gave the highest drug release rate (251.168 mcg/h) followed by E (57.642 mcg/h) and OC (26.887 mcg/h) (Figure 2 and Table IV). Effects of vehicles on 18-β GA release rate were statistically significant ($p < 0.05$). This can be attributed to the low aqueous solubility of 18-β GA [14]. To investigate the state of 18-β GA in vehicles, all formulations were observed using a light microscope using a 20-times magnification (Carl Zeiss Axio Lab.A1, Carl Zeiss, Germany) (Figure 3). The drug was observed to be dispersed in the base gel formulation (G). Influence of penetration enhancers on physical properties of formulations was also investigated in *in vitro* release study. PG addition to the hydrogel (G-PG) gave the highest release rate following the hydrogel without any penetration enhancer (G) ($p < 0.05$). Indeed, PG solved 18-β GA and provided better accommodation for 18-β GA within the polymeric network of the vehicle (G-PG). 18-β GA crystals were not observed clearly under the microscope. However, all hydrogel formulations (G, G-PG and G-L) displayed anomalous 18-β GA transport that can be attributed to two mechanisms conducted drug release, diffusion and polymer relaxation (Table IV). Crystals weren't also detected since 18-β GA dissolved in the inner lipophilic phase of the o/w emulsion and the hydrocarbon vehicle. In that case, increase in the lipophilicity of the vehicle resulted in slower drug release [32, 33]. Here, OA was confirmed

to increase affinity of the drug to the vehicle. It increased the 18-β GA solubility in the emulsion (E-OA) while PG addition generally caused to a higher drug release rate even from OC. L was confirmed to cause slow release rate in the hydrogel vehicle similar to OA (G and G-L, $p < 0.05$) while it did not affect 18-β GA release (E and E-L, $p > 0.05$). Apart from E-PG, 18-β GA release from emulsion formulations (E, E-OA and E-L) were found to fit to anomalous transport mechanism. All oleaginous formulations (OC, OC-PG and OC-OA) displayed 18-β GA release conducted by diffusion.

Table IV

Release parameters of 18-β GA from formulations for 6 hours and kinetic modelling of release profiles

Formulations	Q (mcg/cm^2)	Release rate (mcg/cm^2/h)	Kinetic models								
			Zero order $[Q_t=Q_0 + k_0t]$		First order $[Q_t=Q_\infty(1 - e^{-kt})]$		Higuchi model $[Q_t=Q_0 + k_H t^{1/2}]$		Korsmeyer-Peppas model $[\log [Q_t/Q_\infty] = \log k + n\log t]$		Dominant release mechanism
			r	k_o	R	k_1	r	D	r	n	
G	1504 ± 10.33	251.17 ± 1.73	0.9930	249.80	0.9540	0.35	0.9885	793.05	0.9972	0.83 (non-Fickian)	Anomalous
G-PG	1245.12 ± 35.78	207.94 ± 5.98	0.9948	228.72	0.9467	0.50	0.9820	720.05	0.9949	0.99 (non-Fickian)	Anomalous
G-L	299.31 ± 9.36	49.98 ± 1.56	0.9928	38.26	0.9607	0.22	0.9945	122.23	0.9970	0.51 (non-Fickian)	Anomalous
E	345.16 ± 25.01	57.64 ± 4.18	0.9808	48.53	0.9335	0.25	0.9970	157.32	0.9981	0.60 (non-Fickian)	Anomalous
E-PG	388.78 ± 28.04	64.93 ± 4.68	0.9722	43.76	0.9363	0.17	0.9957	142.92	0.9986	0.41 (Fickian)	Diffusion
E-OA	232 ± 18.20	38.74 ± 3.04	0.9758	33.54	0.8991	0.28	0.9970	109.28	0.9887	0.69 (non-Fickian)	Anomalous
E-L	370.99 ± 17.09	61.96 ± 2.85	0.9937	52.92	0.9518	0.27	0.9969	169.30	0.9991	0.65 (non-Fickian)	Anomalous
OC	161 ± 7.86	26.89 ± 1.31	0.8998	15.59	0.8507	0.14	0.9530	52.67	0.9691	0.36 (Fickian)	Diffusion
OC-PG	317.37 ± 14.68	53.00 ± 2.45	0.9810	24.87	0.9632	0.11	0.9850	79.65	0.9847	0.24 (Fickian)	Diffusion
OC-OA	298.49 ± 8.21	49.85 ± 1.37	0.9953	39.03	0.9651	0.22	0.9948	124.41	0.9951	0.49 (Fickian)	Diffusion

Q: cumulative amount of drug released; Q_t *and* Q_0: quantity of drug released at time t and in the release medium at t=0, respectively;
r: correlation coefficient; k_1, k_0, and k_H rate constants of the Zero order, First order and Higuchi kinetic models, respectively.
Q_t/Q_∞ fractional release of drug, k kinetic constant, and n diffusion exponent of the release mechanism (slope).

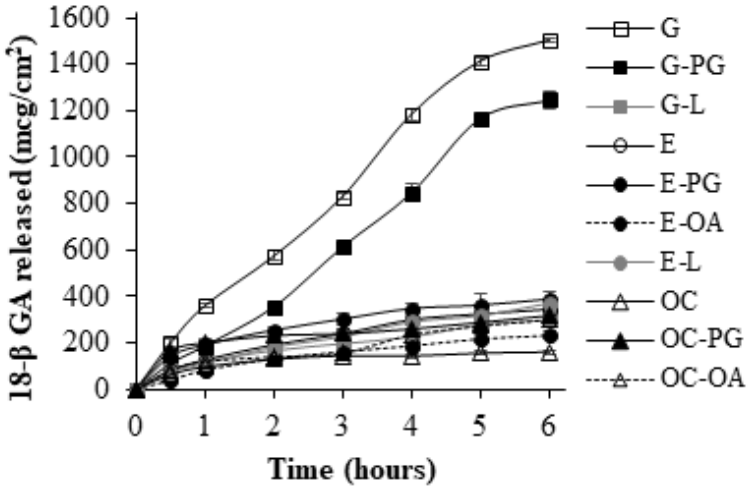

Figure 2.
18-β GA release profiles from the topical formulations in the pH 7.4 PBS:EtOH:PG (2:1:1) mixture

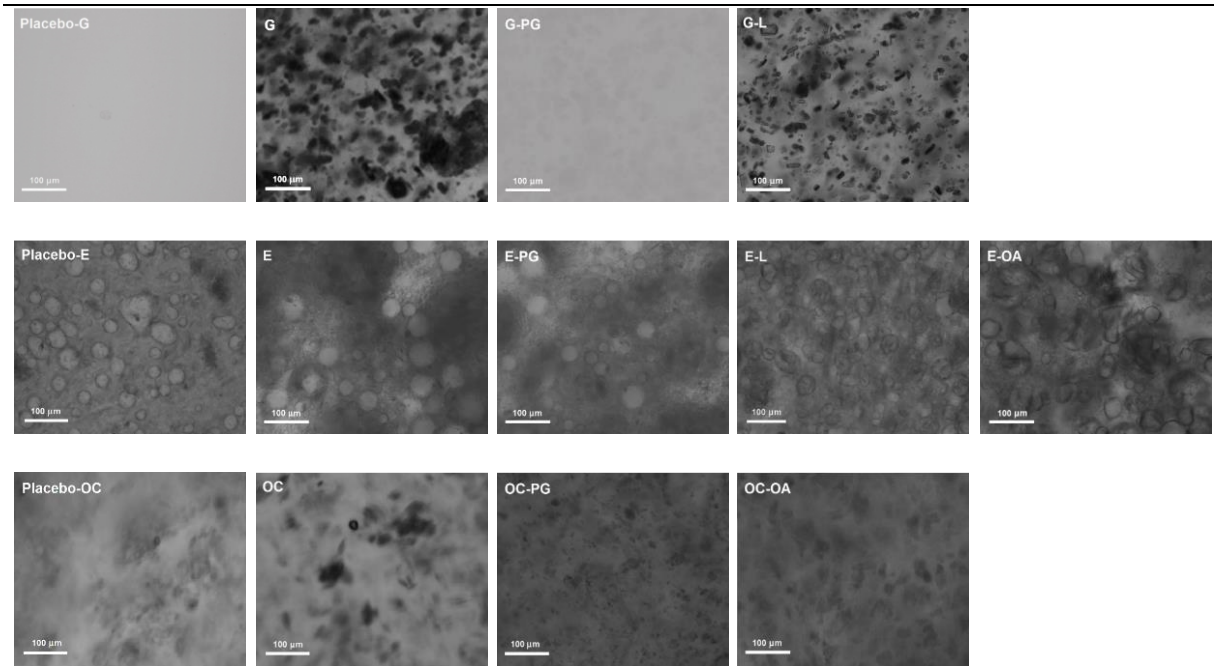

Figure 3.
Light microscope images of formulations (20x)

In vivo assessment of anti-inflammatory effect

The highest oedema inhibition was determined after oral application of the 18-β GA suspension to rats in comparison with topical formulations as we expected (Figure 4) ($p < 0.05$). Anti-inflammatory effect occurred within 1 hour and increased by time. Subsequently, E displayed the highest oedema inhibition followed by OC, when G did not affect to oedema of hind paws of the rats (Figures 5 and 6) ($p < 0.05$). Addition of PG to base formulations gave significantly higher efficacy compared to other penetration enhancers, L and OA ($p < 0.05$). This effect was more obvious in the emulsion formulation (E) ($p < 0.05$). Penetration enhancing effect of OA and L was found statistically insignificant and they were unsuitable penetration enhancers for topical formulations of 18-β GA with different polarity.

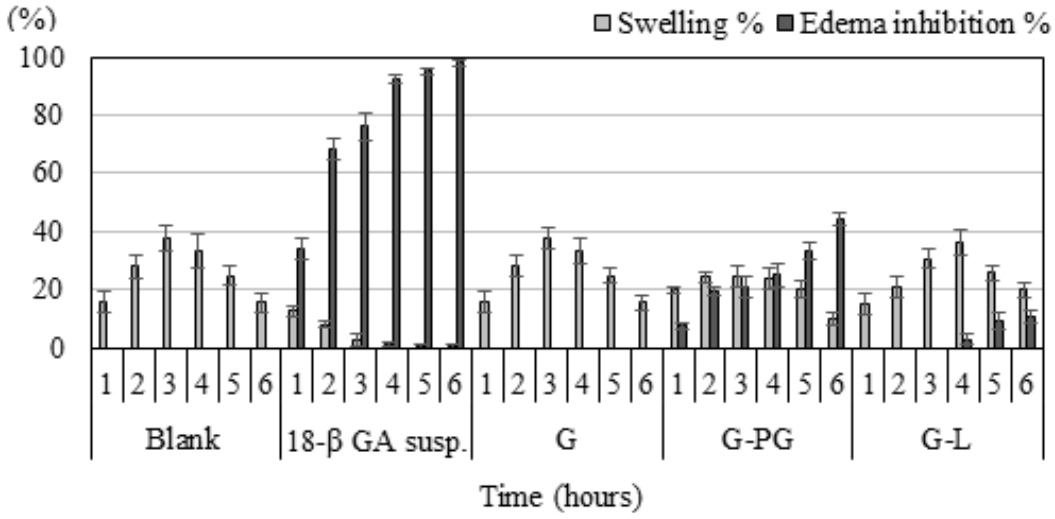

Figure 4.
Swelling % and oedema inhibition % after oral and topical application of the 18-β GA suspension and hydrogels to Wistar albino rats (n = 3)

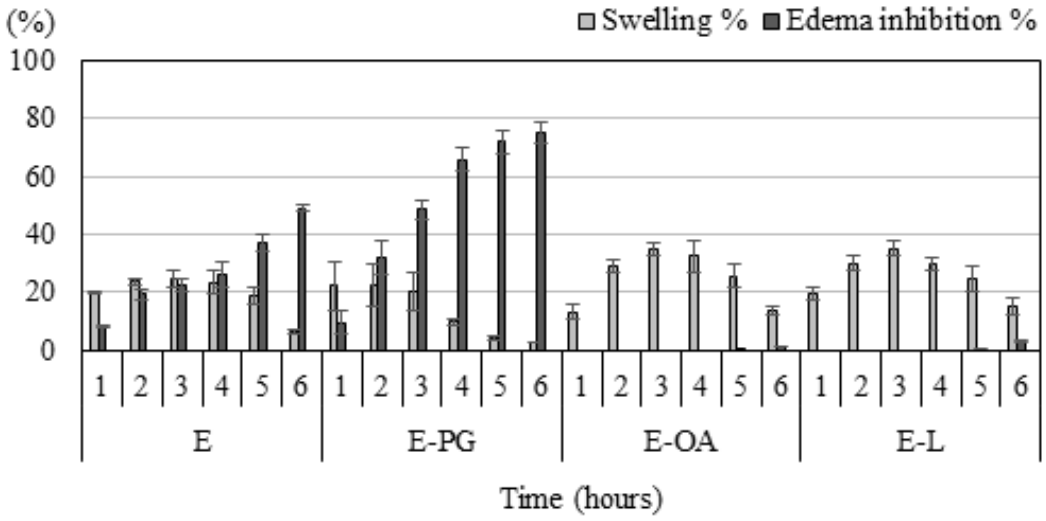

Figure 5.
Swelling % and oedema inhibition % after topical application of 18-β GA o/w emulsions to Wistar albino rats (n = 3)

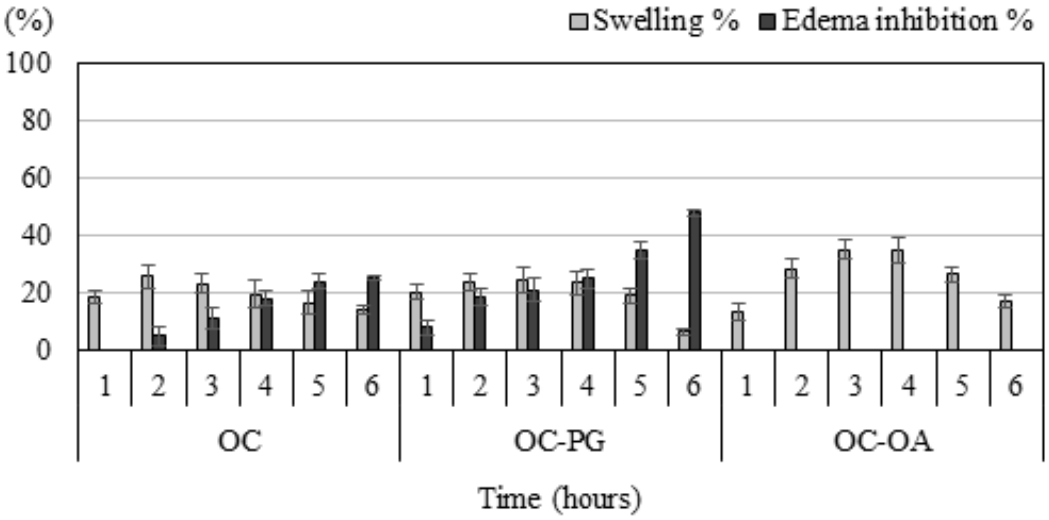

Figure 6.
Swelling % and oedema inhibition % after topical application of 18-β GA oleaginous creams to Wistar albino rats (n = 3)

Ex vivo skin permeation and penetration study

Results obtained from the *ex vivo* skin permeation study were confirmed to be consistent with *in vivo* assessment of anti-inflammatory effect of 18-β GA. Oedema inhibition was determined to be accompanied by drug permeation. 18-β GA permeation from topical formulations through the rat skin was determined to depend on the formulation and type of the penetration enhancer. The highest 18-β GA permeation from base formulations was obtained with E followed by OC and G, respectively (Figure 7 and Table V) ($p < 0.05$). Furthermore, any drug permeation has not observed from G. This can be attributed to the insolubility and the crystalline state of 18-β GA suspended in the hydrogel (Figure 3). If 18-β GA had dissolved in the structure of the xanthan gum hydrogel, the first phase required for transfer of its molecules through a skin pathway would have been completed for penetration process.

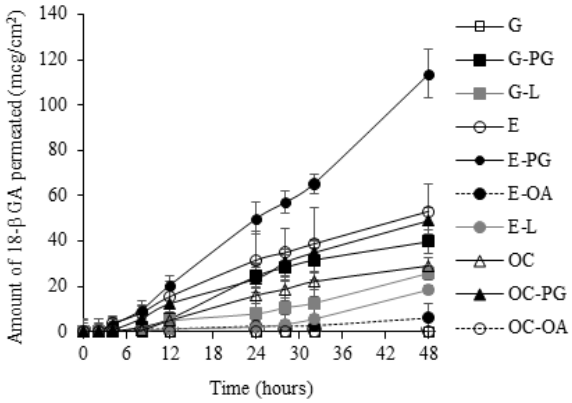

Figure 7.
Penetration profiles of 18-β GA through the abdominal rat skin (n = 3)

Table V

Permeation parameters of 18-β GA through the skin

Formulations	Q_n (mcg/cm^2)	J_s (mcg/cm^2/h)	K_p (cm/h)	Lag time	r
G	0 ± 0	n/a	n/a	n/a	n/a
G-PG	39.81 ± 8.80	1.72 ± 0.31	1.15 x 10^{-4} ± 2.07 x 10^{-5}	4.65 ± 0.58	0.9927
G-L	25.89 ± 5.91	0.75 ± 0.13	0.50 x 10^{-4} ± 0.87 x 10^{-5}	24.12 ± 0.79	0.9970
E	53.19 ± 22.25	1.38 ± 0.39	0.92 x 10^{-4} ± 2.60 x 10^{-5}	4.11 ± 0.24	0.9921
E-PG	113.71 ± 9.55	2.48 ± 0.13	1.65 x 10^{-4} ± 0.87 x 10^{-5}	3.92 ± 0.66	0.9968
E-OA	6.17 ± 6.28	0.19 ± 0.26	0.13 x 10^{-4} ± 1.73 x 10^{-5}	26.08 ± 2.89	0.9919
E-L	18.58 ± 2.32	0.71 ± 0.06	0.47 x 10^{-4} ± 0.40 x 10^{-5}	24.02 ± 0.11	0.9919
OC	29.17 ± 3.85	0.87 ± 0.18	0.58 x 10^{-4} ± 1.20 x 10^{-5}	7.88 ± 0.19	0.9986
OC-PG	49.09 ± 10.67	1.18 ± 0.18	0.79 x 10^{-4} ± 1.20 x 10^{-5}	4.43 ± 0.71	0.9957
OC-OA	0 ± 0	n/a	n/a	n/a	n/a

Q_n: cumulative amount of 18-β GA permeated; J_s: steady state flux of 18-β GA; K_p: permeability coefficient; r: correlation coefficient; n/a: not applicable.

Influence of penetration enhancers (PG, OA and L) was analysed in the respect of 18-β GA permeation. We also attempted to prepare formulations containing isopropyl myristate and Transcutol® as penetration enhancers in addition to PG, OA and L. However, they did not give physically stable formulations at different concentrations. Thus, we decided to continue with PG, OA and L at concentrations that give physically stable formulations (Table II). Previous studies on similar topics also helped us to confirm concentrations of selected penetration enhancers [10, 34]. We also saw that OA and L were physically incompatible with G and OC, respectively. It was found that only the incorporation of PG to vehicles enhanced the penetration rate of 18-β GA up to the 48th h ($p < 0.05$). However, OA and L significantly decreased the permeation rate contrary to our expectations. Addition of PG to formulation E improved drug permeation indicating the most effective formulation (E-PG) followed by E, OC-PG and G-PG, respectively ($p < 0.05$). OA possibly increased the affinity of 18-β GA to the lipophilic phase of E and the lipophilic structure of OC. In the meantime, weakness in the skin barrier caused by disturbing the intercellular lipid packing was insufficient for 18-β GA permeation. Decreased skin/vehicle partitioning of 18-β GA prevented ion-pair formation between drug and fatty acids of the *stratum corneum*. L was also insufficient as a penetration enhancer for 18-β GA. Whereas, PG possibly provided an environment facilitating diffusion of 18-β GA even by modification of the driving force for its diffusion.

Steady-state flux (*Js*) and lag time of formulations were variable as can be seen in Table V. Lag times for E and formulations containing PG (G-PG, E-PG and OC-PG) were earlier (about 4 hours) than other formulations. Formulations displaying permeation followed zero order release kinetics. Amounts % of 18-β GA in the *stratum corneum* and the receptor phase for each formulation after 48 hours are shown in Figure 8. Amounts of 18-β GA extracted from the *stratum corneum* were significantly higher than determined in the receptor phase. These results showed that the amount of 18-β GA penetrated through the skin occurred with E-PG, OC-PG, E and G-PG from the highest to the lowest, respectively. And they were also consistent with the results of the *in vivo* assessment on anti-inflammatory effects of formulations.

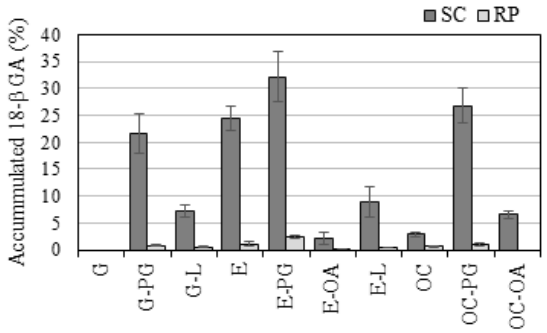

Figure 8.

Cumulative amount of 18-β GA (%) retained in the *stratum corneum* (SC) of Wistar albino rat skins and remained in the receptor phase (RP) after 48 h application of different formulations

Conclusions

It was concluded that *in vivo* and *ex vivo* studies performed on Wistar albino rats provided us information to estimate at least two conditions required for skin penetration of 18-β GA. Highly hydrophilic vehicles are not suitable for transdermal delivery of 18-β GA. Propylene glycol is the most proper penetration enhancer for transdermal delivery of 18-β GA in vehicles with different polarity. Based on the information obtained from the study, the o/w emulsion formulation containing propylene glycol can be recommended for penetration of 18-β GA to alleviate the inflammatory and painful conditions of the skin.

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Conflict of interest

The authors declare no conflict of interest.

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