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Research paper

In vivo investigation, in mice and in man, into the irritation potential of novel amphiphilogels being studied as transdermal drug carriers

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Abstract

Amphiphilogels, gels that consist solely of non-ionic surfactants, are being developed as dermal/transdermal drug delivery vehicles in our laboratories. The irritation potential of two amphiphilogels was investigated on shaved mouse skin, in vivo, and compared to those of Aqueous Cream BP (a moisturiser) and 5% sodium lauryl sulphate (SLS) solution (a known irritant). The skin irritation potential of one of these gels was then investigated in human, using Aqueous Cream BP as a negative control. Skin irritation (following daily application of gels and of controls for 5 days) was assessed by laser Doppler velocimetry, a visual erythema scoring method, and histological evaluations of excised mice skin. We found that the amphiphilogels caused no significant increase in blood flow and in epidermal irritation. In contrast, the SLS solution caused significant perturbation to mouse skin. From this study we conclude that these amphiphilogels may be used as dermal/transdermal drug delivery vehicles.

Keywords: Amphiphilogels; Histological assessment; In vivo skin irritation; Laser Doppler; Surfactants; Visual erythema scoring

1. Introduction

Novel gels, termed amphiphilogels, consisting solely of non-ionic surfactants [1], are being developed in our laboratories for dermal/transdermal delivery of drugs [2]. A range of drugs can be solubilised in the gels [3], with the possibility of delivering them into and through the skin as the surfactants act as penetration enhancers. Before developing the amphiphilogels further, their irritation potential to skin warrants investigation. The skin is comprised of different layers, each with distinct properties and functions. The main role of the outer layer, the epidermis, is to act as a barrier, preventing the ingress of harmful chemicals and microbes into the body, while restricting the loss of water and other ions from the body to the environment. Cells in the uppermost layer, the stratum corneum (SC), are cornified and embedded in a lipid matrix [4,5]. It is this impervious layer that forms the barrier to drug penetration into the skin, as well as providing protection to the body from the external environment. It is this layer, therefore, that will be the first point of contact with any topically applied product. Dermal/transdermal delivery offers a number of advantages such as the avoidance of the first pass effect that usually follows oral delivery [6]; however, the main barrier of the skin must be overcome. The use of chemical penetration enhancers significantly increases the number of candidates suitable for such delivery by increasing skin permeability [7], but a balance between their benefits and adverse effects is needed. Most penetration enhancers interact with skin constituents and might cause reversible or irreversible damage to the skin cells, which must be investigated. Damage to skin cells is a side effect of many, if not all, penetration enhancers, as an increase in permeation cannot be achieved without some perturbation to the skin barrier [8].

Skin irritation is defined as a non-immunological local inflammatory reaction which is usually reversible, and is characterised by erythema and oedema, following a single or repeated application of a chemical to the same cutaneous site [9]. All substances can cause irritation to all individuals, and the factors that determine whether a substance will produce irritation in a certain individual include the age, sex, anatomical site, and other factors particular

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to the individual, as well as the concentration, dose, vehicle, and chemical properties of the irritant, the manner and area of application, and duration of contact. The local inflammatory reaction following perturbation of the epidermal barrier (either by exposure to a physical or chemical irritant) can appear within minutes to hours after the insult, and is believed to be initiated by the release of primary cytokines from keratinocytes [10,11].

There is a range of non-invasive techniques to assess skin irritation or damage, the simplest being visual irritation scoring [12,13], whose main disadvantage is the occurrence of subjective readings. More objective methods such as the measurement of transepidermal water loss (TEWL) and skin blood flow (SBF) using an evaporimeter and laser Doppler velocimetry (LDV), respectively, are required and are widely used in the evaluation of skin irritation [8,14]. LDV is an optical technique that has been used for studying the microcirculation in many tissues, including the skin [15,16]. When a laser beam is directed towards the skin, some of the light is scattered by static structures, and some is scattered by moving objects such as blood cells in capillaries. The light scattered by blood cells is shifted in frequency, and this shift is measured and processed to give parameters relating to the flow of blood cells in the skin. Histological assessment on skin biopsies taken at the end of studies, although invasive, helps to visualise the different layers of the skin and provide more information on skin reactions when evaluating any damaging effects of topically applied preparations [17,18].

In the mid 1940s, Draize et al. [12] published a method for assessing the skin irritation caused by topically applied substances in rabbits, which involves the occluded application of test chemicals to rabbit skin for 24 h duration. Such a method, however, is not considered always reliable for predicting the true irritation potential of topical preparations in man, as demonstrated by Phillips et al. [19]. In addition, pressure from animal rights organisations to eliminate animal testing, has led to increasing efforts to assess the irritation potential of chemicals and topical products in humans in an ethical manner. Shelanski and Shelanski [20] reported a methodology in the 1950s for testing in man; this was followed by a number of other tests [14,21–24] and eventually a protocol was devised by Basketter et al. [25] in 1994 that involved the occlusive application of 0.2 ml (or 0.2 g of solid material) to the skin of the upper outer arm of human volunteers for up to 4 h; treatment sites were then assessed visually for irritation using a 4-point scale at various intervals after patch removal. Other recommendations for the testing of finished products in man were then put in place [26]. Assessment of irritancy of surfactants, or their products, on human skin has a number of inconsistencies; results will depend on the subject group, site, method, and number of applications, as well as on environmental factors, e.g. temperature and humidity. Standardised protocols are required, and although the patch test (devised by Basketter et al.) eliminates a number of the problems mentioned above, it adds the problem of occlusion. This usually blocks diffusional water loss and leads to increased hydration of the skin. This may in itself produce pathophysiological changes in the skin and may exaggerate the irritancy of substances applied topically [27]. It would seem practical and feasible for the assessment of semisolids at least, not to occlude the site of application in human studies, as this will be closer to real-use conditions. The method used in our study has taken into account recommendations from the literature cited above, but has been optimised for the testing of a semisolid preparation such as the amphiphilogel, and does not involve occlusion of the test site.

In this paper, we report on investigations into the irritation effects of amphiphilogels in mice and in man following single and repeated applications. Two gels (20% w/w Span 60 in Tween 20, and 20% w/w Span 60 in Tween 80) were tested in mice and only 20% w/w Span 60 in Tween 80 was tested in man. In previous studies, we have found these gels to be stable at room temperature, and to solubilise some drugs to a high extent [3]. Tween 20 and Tween 80 (fluid phases of the amphiphilogels) are also known permeation enhancers, hence their use in the gels [28,29]. Non-ionic surfactants are believed to have the lowest irritation potential among surface active agents [21,30,31], and only a few instances of irritation have been reported [32,33]. However, Mezei et al. [34] found some skin thickening after daily application of 100% Tween 80 for 10 days, and some epidermal necrosis after a 1-month application in rabbit. When non-ionic surfactants are used in combination, and when they form the bulk of the drug delivery system, such as in our amphiphilogels, their irritation potential might be increased; hence the need for these investigations. In the mouse study, the relative dermal and epidermal tolerance of the gels was compared to that of Aqueous Cream BP, a moisturiser which is not expected to cause any skin irritation, and a solution of sodium lauryl sulphate (SLS), a known irritant [14,30]. In the volunteer study, only the negative control (Aqueous Cream BP) was used.

2. Materials and methods

2.1. Materials

Sorbitan monostearate (Span 60), Polysorbate 20 (Tween 20) and Polysorbate 80 (Tween 80) were obtained from Sigma-Aldrich UK, and used as received. SLS was obtained from BDH UK. Aqueous Cream BP was obtained from Hillcross UK, and used as received.

2.2. Animals

Twenty male T/O mice weighing approximately 20 g (5 weeks old) were obtained from B&K Ltd, Hull, UK.

All animal procedures were conducted in accordance with the Home Office standards under the Animals (Scientific Procedures) Act. The animals were caged in a room with standardised environmental conditions (20 ± 2 °C, 35–45% RH) and a constant day/night cycle. They were fed dehydrated pellets and received water ad libitum throughout the study.

2.3. Volunteers

Fifteen healthy adults (10 men, 5 women), aged 24–55 years (mean 33 years, standard deviation 9) participated in the study after informed consent. Volunteers were free from skin allergies and diseases, and had no history of atopic dermatitis. They were recruited from the School of Pharmacy, University of London, and were given identification numbers upon recruitment. One volunteer dropped out after day 2 of the study due to illness unrelated to the investigation (influenza).

2.4. Formulations

Amphiphilogels with the following compositions were prepared:

Gel 1: 20% w/w Span 60 in Tween 20
Gel 2: 20% w/w Span 60 in Tween 80.

The gels were prepared by heating the appropriate quantities of gelator (Span 60) and solvent (Tweens) in a water bath at 60 °C to produce a clear homogenous sol phase. The latter was left to cool to room temperature overnight and it set to an opaque semisolid gel.

The controls were as follows:

Control 1 (negative control): Aqueous Cream BP (AqC)
Control 2 (positive control): 5% w/v aqueous solution of SLS. 5 g of SLS powder was dissolved in distilled water and made up to 100 ml.

2.5. Animal study

The gels and the negative and positive controls were administered to mice for 5 days; SBF measurements were taken on day 1; erythema was quantified every day throughout the study; skin biopsies were taken at the end of the study and cross-sections of the skin were examined to investigate histological changes, if any, caused by the different preparations. Fig. 1 shows the sequence of events during the 5-day study.

2.5.1. Topical application of preparations

Five mice were assigned to each treatment group as follows: group A received Gel 1, group B received Gel 2, group C received control 1 (AqC), and group D received control 2 (SLS).

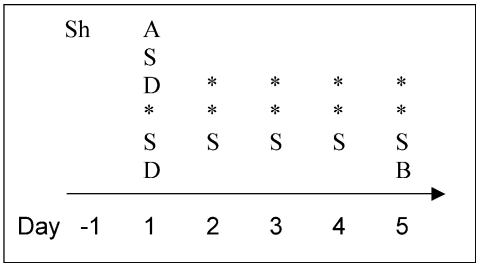

Fig. 1. Mouse schedule that was followed during the 5-day animal study. Sh, shave back with clippers; A, anaesthesia; D, LDV measurement; *, topical application of formulation; S, erythema scoring; B, mice sacrificed and skin biopsy sample taken.

Twenty-four hours prior to the first application, the animals' backs were shaved with clippers, their skin was checked for cuts, and they were allowed to rest overnight. On day 1, a 2 cm^2 patch on their lower backs was marked out for treatment. A similar patch was marked out on their upper backs as the untreated control site. All mice were treated topically with their respective formulations once daily on day 1 of the 5-day study, and twice daily (at 3 h interval) on days 2–5, with an application time of 1 h. 50 mg of gel or cream was applied onto the 2 cm^2 patch on the lower back and carefully rubbed in. For group D, 100 μl of the SLS solution was applied onto the patch. All treatment sites were then covered with sterile gauze and secured with surgical tape to prevent grooming and removal of the formulation from the skin. At the end of the application time, the gauze was taken off and the treated area was gently wiped with water-soaked gauze to remove any residual vehicle from the skin surface. Occlusion was also used as it has been found to produce relatively homogenous and reproducible findings [8,17,35]. However, one disadvantage of occlusion is the possibility that the irritancy of substances may be exaggerated [36,37].

2.5.2. Measurement of skin blood flow by laser Doppler velocimetry

SBF was measured on day 1 of the study before and after topical application of the formulations. The mice were anaesthetised with 0.08 ml Hypnorm (fentanyl citrate 0.315 mg/ml + fluanisone 10 mg/ml) (Janssen Animal Health) intraperitoneal for SBF measurement, as physical movement significantly affects cutaneous blood flow [38]. The LDV measurements were performed using a moorLAB laser Doppler blood flow monitor (Moor Instruments, Devon, UK). Laser safety directions were followed at all times, and the room temperature and humidity were maintained at 20 ± 2 °C and 35–45% RH, respectively, as changes in the environment can also affect cutaneous blood flow and, hence, flux readings [38]. The measuring probe, which contains a laser diode with a wavelength of 780 nm, was fixed gently onto the skin with an adhesive tape to avoid vascular compression. The readings (flux in arbitrary units, au) were taken for 5 min after stabilisation of the output signal. Measurements of the application site

prior to topical application provided a baseline value for untreated, non-irritated skin. After removal of the formulation, the skin was allowed to rest for 10 min before any SBF measurements were taken. For each mouse, an irritation index was calculated, where the absolute values are normalised with respect to the corresponding pre-treatment values, as follows [39,40]:

$$\text{Irritation index} = \text{mean FLUX after application/mean FLUX before application}$$

2.5.3. Visual assessment of skin irritation

The skin irritation (erythema) was evaluated after each application according to the following scale: 0, no erythema; 1, slight erythema (barely perceptible, pink); 2, moderate erythema (dark pink); 3, moderate to severe erythema (light red); 4, severe erythema (extreme redness) and/or other effects, e.g. fissures [8,12].

2.5.4. Histological assessment

At the end of day 5, the animals were sacrificed and 1 cm^2 skin biopsies from all treated and untreated sites were taken and preserved in 10% formalin solution for at least 48 h. Following dehydration through a graded series of alcohols and embedding in paraffin wax, sections of 4 μm thickness were cut from each sample, parallel to the direction of hair growth, stained with haematoxylin and eosin (H&E) and examined with a light microscope [17,41]. Each sample was scored according to a system modified by Lashmar et al. [17] from Ingram and Grasso [41]. Each cross-section sample was photographed at the midpoint and halfway to the edge on either side of the midpoint, and scored to give an average for each biopsy sample. Parameters such as thickening of the epidermis, thickening of the SC (hyperkeratosis), and infiltration of the dermis, were examined and their intensity scored (see Table 1). Mean histological score was then calculated for each treatment as follows:

$$\text{mean score} = \frac{\sum Aa + \sum Bb + \sum Cc + \cdots}{n}$$

where A, B, C, etc. are the scored histological parameters, e.g. hyperkeratosis, a, b, c etc. are the frequency of occurrence of a particular score for a histological parameter in any one group, and n is the group size. A score of <10 was regarded as absence of undue reactions to mouse skin, and a score of >21 was regarded as unacceptable damage [17].

2.6. Human study

The skin irritation potential of one amphiphilogel (GEL 2) was investigated in a single-centre, randomised, controlled, semiblinded, intraindividual comparison study, in healthy volunteers with normal skin. The study was reviewed and approved by the Camden and Islington Local Research Ethics Committee. The investigation protocol is shown in Fig. 2.

2.6.1. Application regimen

The study was conducted over 4 weeks (up to four volunteers per week), and the volunteers received both study treatments. The gel (50 mg) was rubbed into a hairless site (diameter 3 cm) on one forearm. It was applied once for 1 h on the first day of the study (to test single insult challenge), then twice daily (1 h duration at 3 h intervals) for the next 4 days (to test repeated insult challenge). The same procedure was conducted on the other forearm using the control—50 mg of Aqueous Cream. It was important to follow the same procedure of application as irritation results can depend on the method and site of application, as well as on environmental factors. Occlusion was not used because the applied preparations were semisolids and the method of application closely resembled application of topical products in normal usage. Before any assessments or measurements were made, the site of gel/cream application was carefully wiped with a damp tissue to remove any residual formulation from the skin.

The gel was applied to the right arm of volunteers with an odd subject identification number, and to the left arm of those with an even identification number. This provided some form of randomisation, known only to the investigator who applied the formulations. Blinding of this investigator could not be achieved as the gel and the cream differ in appearance. In contrast, the investigator who visually assessed skin erythema was blinded as to which preparation was applied to which forearm, to prevent any bias.

The volunteers were allowed to bathe as usual, but were asked to avoid direct application of detergents, moisturisers, emollients, or other topical preparations on their forearms during the 5-day study. Before any LDV measurements, the volunteers completed a questionnaire in which they recorded any food and drink they had had in the previous 12 h, as well as any adverse effects experienced. It is known that certain foods and drinks can alter SBF [38], and a record of the food and drink consumed is therefore important in case of anomalous results.

2.6.2. Visual assessment of cutaneous irritation (erythema scoring)

The site of gel/cream application was visually examined for cutaneous irritation/reaction and a score for erythema (redness) and oedema (swelling) was given as follows: 0, no reaction; 1, weak spotty or diffuse erythema; 2, weak but well perceptible erythema covering the total exposure area; 3, moderate erythema; 4, severe erythema with oedema; 5, very severe erythema with epidermal defects (vesicles, erosions, etc.). Such quantification of erythema and oedema was similar to that used by Frosch et al. [42]. All the visual assessments of erythema were measured by the same researcher (not the investigator who applied

Table 1
Histological score frequencies for each group

Parameter and score		Frequency of scores with:			
		Gel 1 ($n = 5$)	Gel 2 ($n = 5$)	AqC ($n = 5$)	SLS ($n = 5$)
Epidermal changes					
(A) Epidermal thickening					
No change	0		1	3	N/A
2 × normal in places	1	2		2	
2 × normal generally	2	3	4		
(B) Increase in cell layers of stratum granulosum					
No change	0			2	N/A
By 1 cell layer	1	1	1	3	
By 2 cell layers	2	2	3		
By 3 cell layers or more	3	2	1		
(C) Hyperkeratosis (thickening of stratum corneum)					
No change	0			1	N/A
Mainly loose	1	2	4	4	
Mainly severe	2	3	1		
(D) Spongiosis					
No change	0	1	5	5	N/A
Slight	1	4			
(E) Intracellular oedema					
None	0	5	5	5	N/A
Present	1				
(F) Destruction of epidermis					
None	0				
Superficial	15				2
1/4 of sectioned area	18	N/A	N/A	N/A	
1/2 of sectioned area	20				2
3/4 of sectioned area	25				1
Dermal changes					
(G) Hyperaemia (increased blood cells in dermis)					
None	0	5	5	5	2
Slight	5				3
(H) Increase in density and thickness of collagen					
Bundles	0	5	4	5	1
None	1		1		1
Slight	2				2
Slight to moderate	3				1
(I) Fractured collagen					
None	0	5	5	5	4
Slight	1				1
(J) Infiltration of the dermis					
None	0	4	3	4	
Slight in the uppermost layer	1	1	2	1	
Slight diffuse	2				
Moderate in the uppermost layer	3				
Moderate diffuse	4				2
Severe in the uppermost layer	5				2
Severe diffuse	6				1
Mean histological score (calculation shown in method)		6	5	2	29

N/A, parameter not scored; Gel 1, 20% w/w Span 60 in Tween 20; Gel 2, 20% w/w Span 60 in Tween 80; AqC, Aqueous Cream BP; SLS, 5% w/v SLS in water.

the preparations), prior to and independently of, the blood flow measurements. The median erythema score of all the volunteers was used as the value for each formulation on each day. It was decided that if the erythema score developed to a severe degree (≥ 3) for a volunteer, application of the formulation would be discontinued for that volunteer and the maximal erythema scores would be used on all subsequent days.

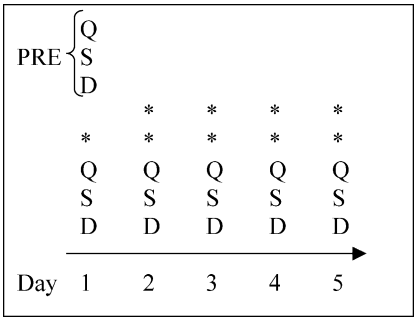

Fig. 2. Schedule that was followed by volunteers during their 5-day study. Questionnaires before the LDV measurements were used to note down any changes since the last LDV measurement, e.g. meals, drinks, any untoward reactions, etc. PRE, pre-application on day 1; Q, questionnaire; S, visual erythema assessment and score; D, LDV measurements; *, topical application.

2.6.3. Measurement of skin blood flow by laser Doppler velocimetry

Cutaneous blood flow at the site of application was measured prior to gel/cream application on day 1 and at the end of each day of the study period (1 h after the last application of the day) using the moorLAB laser Doppler blood flow monitor. Before measurements were taken, the volunteers rested for approximately 15 min in the investigation room to allow cutaneous blood flow to reach a resting state. During measurements, they were seated comfortably, with arms outstretched on armrests. The measuring probe was gently fixed onto the skin with adhesive tape and readings (flux, in arbitrary units) were taken for 5 min after stabilisation of the output signal. This was carried out three times, with 5 min intervals, and the mean flux value was used. Flux readings in the two arms were measured alternately. Mean flux values for each treatment group was measured as the mean of all the flux values of the volunteers for that particular treatment. LDV values of treated forearm sites were also compared to LDV values obtained before any application, and an irritation index was calculated as described in Section 2.5.2.

It was decided that if volunteers discontinued the study due to irritation, with an erythema score of ≥ 3, the measured flux values obtained on the day of withdrawal from the study would be used for the final calculations.

2.7. Data analysis

Statistical evaluation of the SBF data was performed using the Student's *t*-test. Erythema scores were evaluated by the Wilcoxon and Mann–Whitney *U*-tests (5% significance level). The histological results (as frequencies of positive and negative effects) were evaluated statistically for each parameter using the Fisher exact test for 2×2 contingency tables ($\alpha = 0.05$).

3. Results and discussion

It is important to evaluate the irritancy potential of any topical preparation in order to assess the extent of damage that it may cause to the skin, and decide if this is acceptable. To quantify skin irritation caused by the amphiphilogels in mice and in man, we used the method of visual scoring of erythema (a subjective method that is rapid, easy, and widely used) in addition to the objective bioengineering technique of LDV, and histological assessments of treated mouse skin.

3.1. The gel formulation

The amphiphilogels are prepared by a very simple method; when the gelator and the fluid phase are mixed and heated, a clear isotropic solution, known as the sol phase, is formed. Upon cooling, the sol phase sets to a smooth opaque semisolid; this is due to a fall in the solubility of the gelator (Span 60) in the liquid medium, and a reduction in the solvent–gelator affinities. As a result, Span 60 molecules self-assemble into a three-dimensional network that entraps the fluid phase and immobilises it. Thus a gel is formed. The gel is thermoreversible, i.e. upon heating, the gel melts to the sol phase that can be cooled again to the gel state [1]. Light microscopy on the gels reveals the presence of tubules and clusters of tubules of diameter ≈ 40 μm (Fig. 3). These clusters are most probably aggregates of small tubules of self-assembled gelator molecules. Various drugs can be incorporated into the gels before topical administration for a local or systemic action. The surfactants present in the gels are expected to aid delivery of the drugs through the skin by acting as skin permeation enhancers [28,29].

3.2. Mouse study

3.2.1. Skin irritation measured by LDV

SBF measurements were conducted before and after a single topical application of the gels and of the controls on

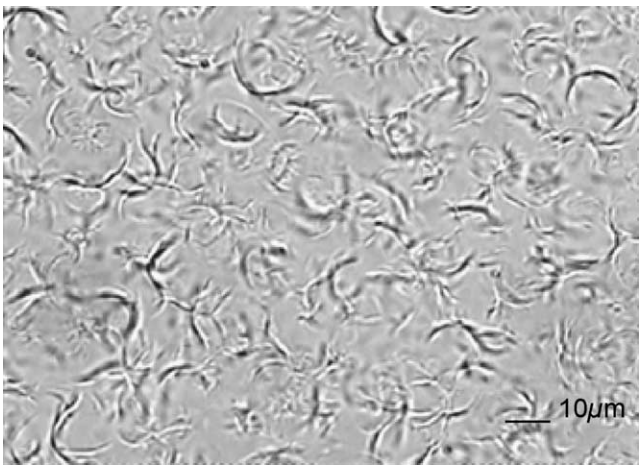

Fig. 3. Light micrograph of a 20% w/w Span 60 in Tween 80 amphiphilogel.

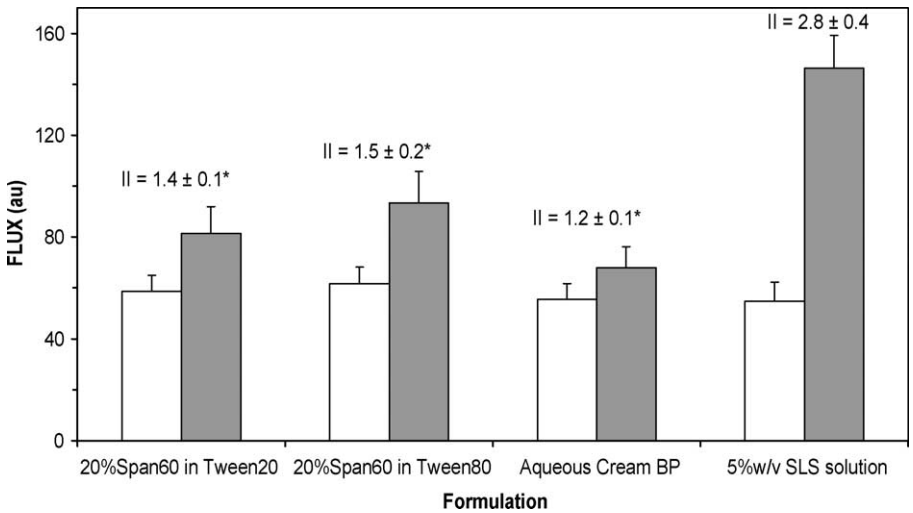

Fig. 4. Skin blood flow measurements of mouse skin before (white) and after (grey) occlusive application of the formulations. The irritation index (II) (SBF readings after exposure/SBF readings before exposure) is shown on top of the bars. *$P < 0.05$ compared to treatment with SLS.

day 1 of the study (as per schedule in Fig. 1). Flux (before and after topical application) and the degree of irritation caused by the different formulations, expressed as an irritation index (II), are shown in Fig. 4. There was a significant difference in SBF before and after exposure to 5% SLS (reference irritant) ($P < 0.01$). Treatment with the amphiphilogels also caused an increase in SBF ($P < 0.05$); however, the irritation indices of the gels were not found to be significantly different to that of the Aqueous Cream BP ($P > 0.05$), and the three formulations gave an irritation index between 1.2 and 1.5. On the other hand, the irritation indices of the amphiphilogels were significantly different from that of SLS, which caused almost a three-fold increase in SBF ($P < 0.05$). The results show that the gels are no more perturbing to mouse skin than Aqueous Cream BP, and are considerably less irritating than a solution of 5% w/v SLS following a single 1 h occlusive application. The slight increase in SBF following application of the gels may be an advantage for transdermal delivery, where the drugs passing through the skin barrier can be carried away more efficiently by the increase in blood volumes and speed, while at the same time irritation to the skin is not excessive.

3.2.2. Skin irritation measured by visual erythema scores

Erythema was evaluated visually after each exposure of the mouse skin to the different formulations. Erythema, which is caused by increased blood flow in the dermis, enables us to monitor the response of that layer to topical preparations. The scores for each mouse at the end of days 2, 3, 4 and 5 are shown in Fig. 5 to give an indication of the changes in erythema with repeated applications. All scores on day 1 were zero for all formulations. Aqueous Cream BP did not cause any visible changes on any day, to any mouse. 5% SLS solution, however, caused irritation in the form of erythema and wrinkling that was visible from day 2. The irritation increased further each day and produced fissures on the skin surface of some of the mice by day 5. Under occlusion in other studies, SLS preparations with concentrations as low as 5% w/v were found to cause inflammation and erythema [14,44] and significant changes in skin morphology [18,41]. Following repeated applications of both amphiphilogels, erythema increased slightly; this occurred more frequently in the group treated with Gel 2. This reflects the slightly higher irritation index of Gel 2 shown in Fig. 4. However, no statistically significant difference could be found between the erythema scores before treatment and on any day after treatment with the gels, at the 5% level (Wilcoxon test). There was no significant difference between the erythema scores of Gel 1 and Gel 2; and between either gel and Aqueous Cream BP, on any day (Mann–Whitney *U*-test). In contrast, the erythema scores for the gels were significantly lower than those produced by the SLS solution from day 2 onwards ($P < 0.05$). The median scores for each group, as well as the statistical significance compared with SLS, are shown in Fig. 5 under the group names. From these observations, we conclude that the amphiphilogels are significantly less irritating compared to a 5% SLS solution, and have a similar irritation potential to Aqueous Cream BP, a widely used moisturiser.

Our results show a good correlation between LDV measurements and visual scoring of erythema. This is to be expected as both methods measure increased blood flow; a positive relationship between the two has also been reported previously [45,46]. LDV has the advantage of being an objective method, does not rely on an observer's estimation of redness and is a more sensitive method [8,14,43]. For example, irritation was detected by the LDV after a single occlusive application of 5% SLS solution, even though no erythema could be seen at the end of day 1. LDV is thus a useful tool to measure mild to moderate irritation, and has been successfully used to measure the irritation potential of a number of chemicals, in addition to visual scoring [8,14,43].

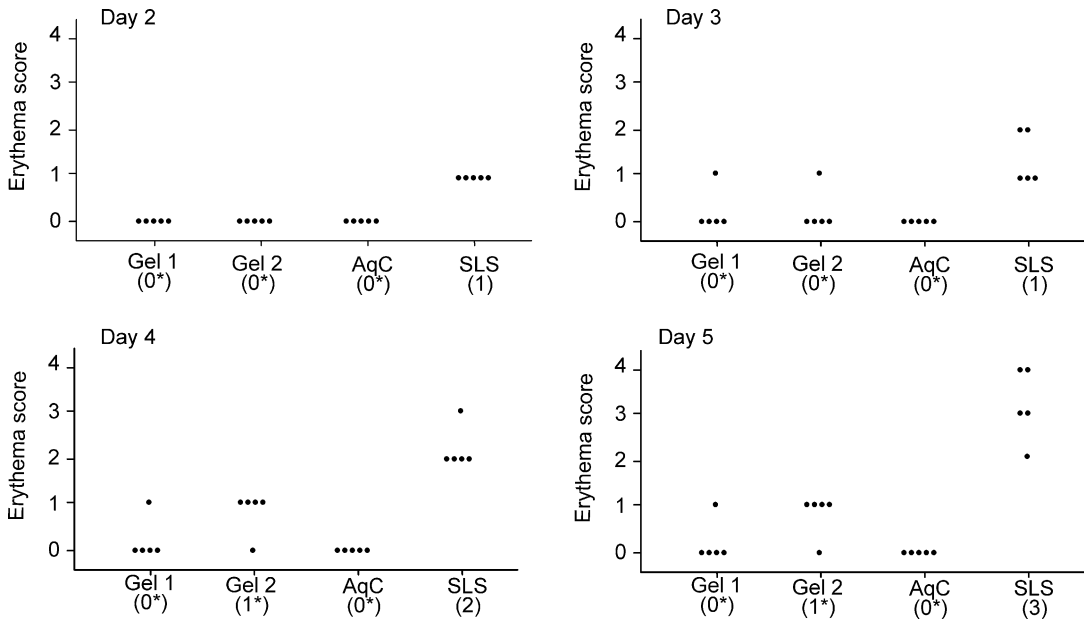

Fig. 5. Erythema scores for each mouse (represented by •, $n = 5$) at the end of days 2–5 following twice-daily application of each formulation. All erythema scores were zero at the end of day 1. The numbers in parentheses indicate median erythema score for the group. *$P < 0.05$ compared with SLS solution.

3.2.3. Histological assessment of skin biopsies

At the end of the study, skin biopsies were examined microscopically and histological scores (quantifying the damage caused the application) were calculated (Table 1). Histological assessment allows one to quantify the damage caused to the epidermis and SC, such as swelling of the individual cells (intracellular oedema). Each sample was assessed and scored according to the changes observed for a number of histological parameters, e.g. in group A (mice treated with Gel 1), two biopsy samples were given a score of 2 for parameter B, where the number of cell layers in the stratum granulosum of the epidermis increased by two cell layers. Dermal damage, observed as an increase in blood vessels and as changes in the collagen and elastic fibres (parameters G–J), indicates the penetration of the irritant into this layer of the skin, once the SC has been damaged [22]. The overall severity of the microscopic findings for the gels and Aqueous Cream was mild to moderate (mean histological score <10), while that of SLS solution was much more severe, with a mean score of 29. These scores agree with the LDV and erythema measurements, discussed earlier, which showed similar irritation potentials of the gels and of Aqueous Cream and a significantly higher irritation caused by SLS. The mean histological scores are also reflected in the sample micrographs shown in Fig. 6(i)–(v). Fig. 6(i) shows that normal, non-irritated mouse skin has a thin epidermis with a thin and wavy SC, and the stratum granulosum is one cell-layer thick, if visible at all. Aqueous Cream, being the least irritant, caused the least change to the skin, with only some hyperkeratosis (thickening of SC) (Fig. 6(iv); Table 1); followed by the amphiphilogels, which caused some epidermal thickening (most common effect), increase in cell layers of the stratum granulosum, hyperkeratosis, rarely spongiosis (intercellular oedema), and slight infiltration of the dermis (Fig. 6(ii) and (iii); Table 1). The reference irritant SLS caused much damage, including destruction to the epidermis, pronounced hyperkeratosis, parakeratosis (persistence of nuclei in SC), spongiosis, and hyperaemia (increase in blood vessels) in the dermis, as well as other dermal effects (such as changes in the nature of the collagen) (Fig. 6(v); Table 1). Such pathological changes caused by SLS have been reported previously [18,30,35,40], and are thought to be related to the surfactant's ability to bind to, and denature, epidermal keratin. In contrast, the non-ionic surfactants, such as those making up the amphiphilogels, have been found to cause little damage [21,30]. As these surfactants lack any charged groups in their structures, they are unable to interact well with keratin and, thus, are less likely to cause significant damage to the skin [30,47].

3.3. Human study

3.3.1. Erythema scores

The sites of gel (Gel 2) and cream (AqC) application of all volunteers were assessed visually before and after applications, prior to the SBF measurements, as shown in Fig. 2. The erythema scores for all volunteers were zero at all times, for both formulations (Table 2). It seems that the amphiphilogel does not cause any irritation to the skin following a single insult (result on day 1) and following repeated challenge over a period of 5 days.

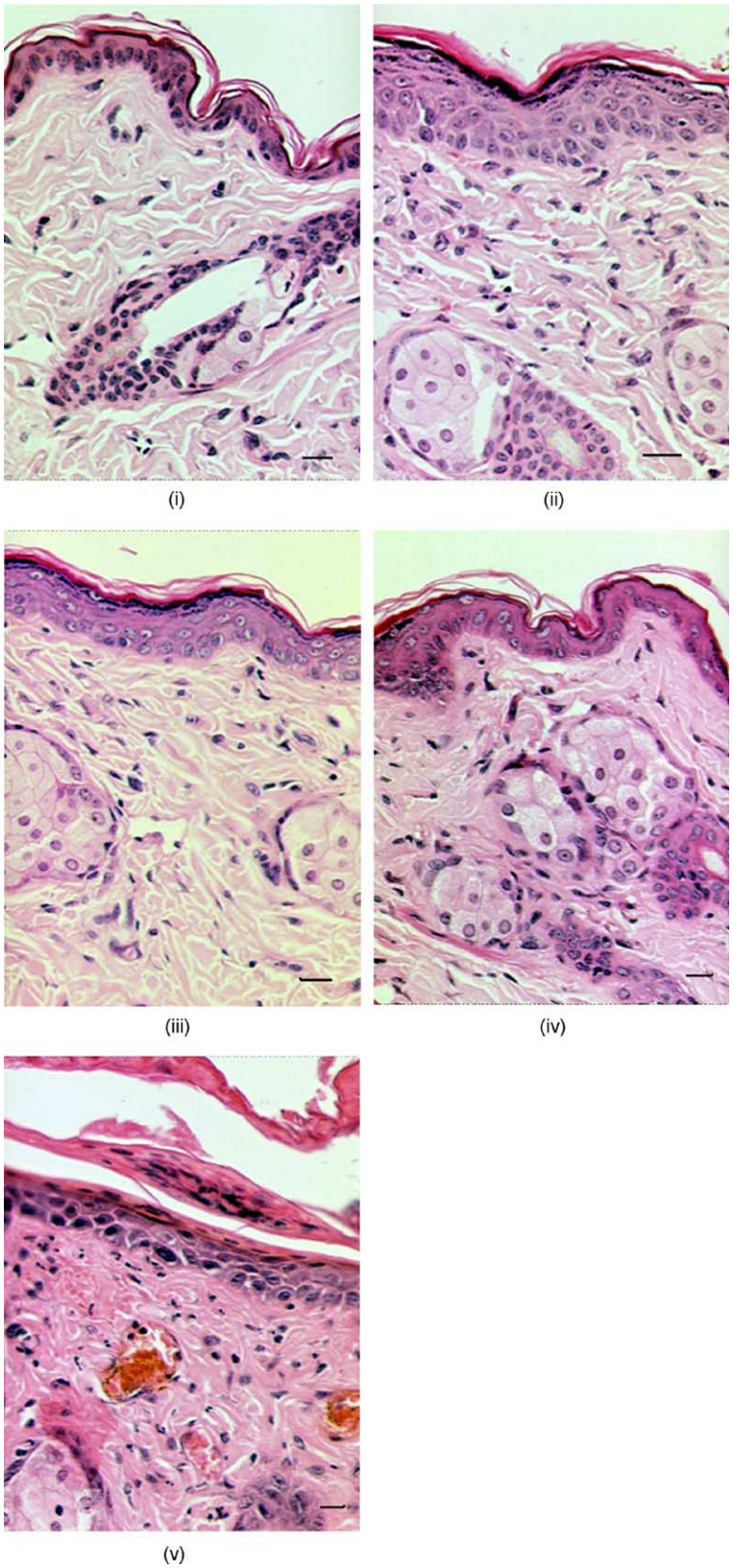

Fig. 6. Histological photomicrographs of skin biopsies taken from mice treated with various formulations. H&E stain. Bar 10 μm. (i) Untreated mouse skin showing thin stratum corneum, distinct boundary between epidermis and dermis, and hair follicle base. (ii) Mouse skin treated with 20% w/w Span 60 in Tween 20 gel for 5 days, showing an increase in the thickness of the epidermis. (iii) Mouse skin treated with 20% w/w Span 60 in Tween 80 gel for 5 days, showing an increase in the thickness of the epidermis. (iv) Mouse skin treated with Aqueous Cream BP for 5 days, showing no significant change in the skin. (v) Mouse skin treated with 5% w/v SLS solution for 5 days, showing considerable damage to the epidermis, with hyperkeratosis and intercellular oedema.

Table 2
Median erythema scores for all the volunteers following application of either the Aqueous Cream BP (AqC) or amphiphilogel (Gel 2)

Application of:	Median erythema score					
	Pre ($n = 15$)	Day 1 ($n = 15$)	Day 2 ($n = 15$)	Day 3 ($n = 14$)	Day 4 ($n = 14$)	Day 5 ($n = 14$)
AqC	0	0	0	0	0	0
Gel 2	0	0	0	0	0	0

PRE, pre-application values on day 1.

3.3.2. Skin blood flow measurements by LDV

The SBF measured by LDV, following application of the gel and the cream for each day is shown in Fig. 7. The relatively large standard deviations observed could be due to a number of factors such as physical movement of the volunteer, or location of the probe, although measures were taken to ensure that the volunteers were suitably relaxed prior to SBF measurements and that the probe was placed in the same position each time. However, it must be appreciated that even a slight shift in the location of the probe can have a significant effect on the flux readings, depending on the density of blood capillaries present within the dermis under the probe.

No significant difference could be found between any of the post-application values and the corresponding pre-treatment flux values, except for the gel on day 4 ($P < 0.05$). The mean irritation indices (ratio of post-treatment flux to pre-treatment flux) of the cream and gel were calculated for each day (Table 3) and were found to be similar. No statistically significant difference could be found on any day between the irritation indices of the gel compared to those of the Aqueous Cream BP ($P > 0.1$). This indicates that the amphiphilogel does not perturb the skin (any more than Aqueous Cream BP) when applied daily for 5 days.

Any irritation that may have occurred by day 4 seems to have subsided by day 5. Looking at the individual flux data, it seems that the higher flux values on that day were measured from volunteers who took part in the same week of the investigation, where day 4 was a particularly warm day (26th June 2003) and volunteers have been enjoying the warm weather, which may have affected the SBF, despite our attempts to maintain the investigation room temperature constant. SBF on the cream-treated arms was also quite high on day 4, but not significantly different to pre-treatment values. Although the irritation indices of the gel and the cream vary, they were not significantly different from one another, which indicates that it may have been another factor that had led to the increased SBF, such as the warm weather, as mentioned above.

Once again LDV and erythema scoring, gave the same result; that is, the amphiphilogel does not cause any skin irritation when applied daily for 5 days. The non-invasive biophysical technique of LDV proved to be a simple, easy method for the user, comfortable for the volunteers, and produced quantitative data. It may seem more sensitive and objective than visual assessment for erythema, but both methods led to the same conclusion.

3.3.3. Single insult challenge

The results obtained on the first day of the study give an indication of the possible effects of the gel following a single application. As can be seen from Table 2, no visible erythema was observed. This concords with the LDV

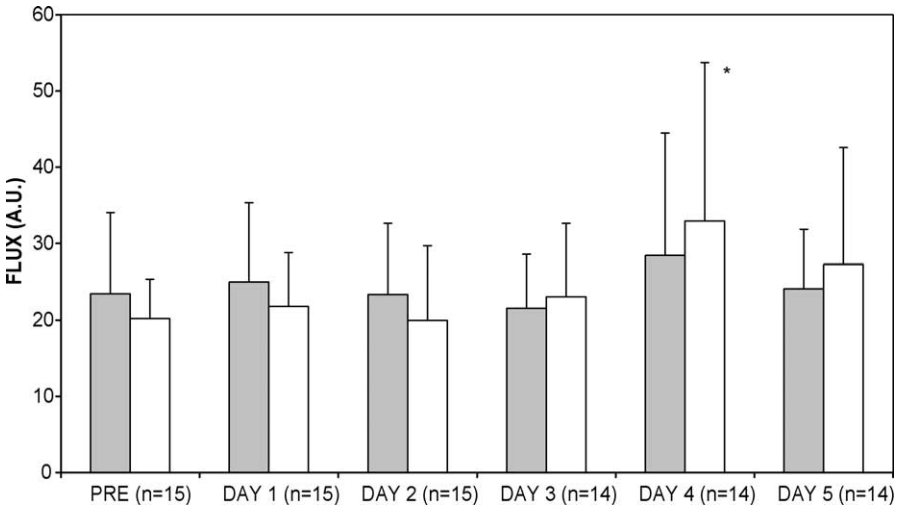

Fig. 7. Mean skin blood flow values before and after daily application of Aqueous Cream BP (grey) and amphiphilogel (white). PRE, pre-application. *$P < 0.05$ compared to pre-application (PRE).

Table 3
Mean irritation indices for all the volunteers following treatment with either the Aqueous Cream BP (AqC) or (20% Span 60 in Tween 80) amphiphilogel (Gel 2)

Application of:	Mean irritation index ± standard error				
	Day 1 ($n = 15$)	Day 2 ($n = 15$)	Day 3 ($n = 14$)	Day 4 ($n = 14$)	Day 5 ($n = 14$)
AqC	1.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.3 ± 0.2	1.1 ± 0.1
Gel 2	1.1 ± 0.1	1.0 ± 0.1	1.3 ± 0.2	1.7 ± 0.2	1.4 ± 0.2

measurements. The mean irritation indices (± standard error) following the first application of gel or cream are both 1.1 ± 0.1, and no significant difference exists between the two (see Table 3). We can conclude that a single application of the amphiphilogel, for example when the gel is used to administer a vaccine, is unlikely to cause irritation to adults.

3.3.4. Repeated insult challenge

The results for the week-long study, especially those on day 5, give an indication of the cumulative effect of gel application onto the skin. Irritation was assessed daily to detect changes in blood flow over time and to identify any adverse effects before they developed further. According to the erythema scores (which were all zero throughout the week), the gel and the cream caused no cumulative irritation. In contrast, the SBF results show some increase in cutaneous blood flux with time, especially towards the end of the week with the gel application. Blood flux following cream application also increased with time, and the mean irritation indices of the two formulations did not differ significantly on any day. The results indicate that the gel does not cause any more irritation to the skin, compared to the negative control, Aqueous Cream BP, which is a moisturiser and is used to treat skin irritation and dry skin conditions.

It is encouraging to find that the amphiphilogel, which contains such a high concentration of surfactants did not cause any irritation when applied on five consecutive days to human skin. The volunteer study concords with skin irritation studies carried out in mouse which indicated a slight, but not statistically significant (compared to Aqueous Cream BP) irritation to mouse skin by the amphiphilogel. The findings of this study are in agreement with those of Treon [51], who reported no adverse reactions to 100% Tween 80 when applied topically to 50 human subjects. The surfactant was kept in contact with the skin for 72 h; this was repeated after 7 days, before assessing the skin for oedema and erythema.

Our results also concord with studies (where mixed surfactant solutions were occlusively applied topically for 4 h) which indicated that the irritation potential of mixed surfactant systems was lower than the additive irritation potential of the two individual components [48,49]. The lower irritation was explained by a reduction in CMC of the mixed surfactant system and reduced surfactant monomer concentration, the monomer being the species which binds to and denatures keratin in the SC.

There have been a few irritancy studies in animal using Tween 20, the major constituent of Gel 1; Mezei et al. [34] found erythema, oedema, and skin thickening when Tween 20 was applied daily to rabbit skin for 10 days; however, Tavss et al. [50] found no irritation to human subjects after 5-day exposure to 10% aqueous solution of Tween 20, compared to a severe irritation within 1 day when a 10% SLS solution was applied. In vitro tests using human skin showed a 0.2% increase in swelling of the SC after 1 h incubation with 2% w/v Tween 20, compared to a 32% change with SLS [47]. No SC swelling was found with Tween 20-treated skin in another study [31]. The pathological changes caused by Gel 2 (20% w/w Span 60 in Tween 80) are similar to changes caused by Tween 80 [30,34]. This is to be expected considering that Gel 2 is made up predominantly of Tween 80. Mezei et al. [34] observed erythema and oedema after 3 days of topical application of 100% Tween 80 to rabbit skin; irritation increased with duration of application and pronounced histological changes such as acanthosis (increase in thickness of stratum spinosum) and necrosis were observed after 10 days and 1 month, respectively. Our results only indicated slight erythema and epidermal thickening in some of the mice after 5 days of exposure to the gel.

It is particularly important to compare our results with the data produced by Mezei et al. [34] who studied the effects of non-ionic surfactants at concentrations up to 100%. In contrast to our findings, which showed little irritation to mouse skin caused by the gels after 5 days of applications, Mezei et al. concluded that non-ionic surfactants were irritating to the skin. In their studies, various non-ionic surfactants were applied daily, for up to 30 days, onto rabbit skin. They also found that Span 60, dissolved at up to 60% w/w in petrolatum base, caused slight erythema after 10 days application. Results by Mezei et al. were also found to be in conflict with other studies conducted at around the same time, which indicated that only mild irritation, if any, was caused by the non-ionic surfactants tested by Mezei et al. [30,31,51]. It is possible that the conflicting results are due to different methods and durations of application, as well as different concentrations of surfactants used and different methods of evaluation of damage.

Skin irritation is a complex mechanism involving epidermal and dermal cells interacting with one other and with blood cells, under the influence of cytokines generated

mainly by the epidermal keratinocytes that have been activated following the application of an irritant, or a physical perturbation to the epidermal barrier [52]. This usually leads to cutaneous vasodilatation and is manifested by erythema and local swelling. The exact mechanism of surfactant-induced skin irritation is not fully understood and a number of hypotheses have been suggested. Irritation may be caused by binding of the surfactants to keratin in the SC, via ionic and/or hydrophobic interactions, and by concomitant protein denaturation resulting in swelling of the SC [47,53], or it may be due to lipid removal from the SC which exposes more binding sites on the keratin [53]. Removal of lipids leads to irreversible loss in SC structure; hence, reversible damage to the skin by surfactant is thought to be due to its interaction with the horny layer proteins [47,54]. Non-ionic surfactants carry no charges; therefore, they should not interact ionically with ionic proteins in the skin, which may explain their lower irritancy potential. However, they are capable of forming hydrogen- and hydrophobic-bonds with skin components, especially lipids, and thus, can modify the skin's physical properties. It has also been found that non-ionic surfactants' interaction with, and disruption of epidermal membranes, results in an increase in phospholipid biosynthesis in order to regenerate the original membrane structure [55–57].

It has been suggested that surfactant monomers are involved in skin irritation, with the greatest irritation caused by surfactants with an alkyl chain length of 12–14 carbons in a homologous series, which corresponds to the maximum absorption of the surfactants into the skin [47,53]. This is probably due to optimal binding between hydrophobic regions on the proteins and surfactants containing such alkyl chain moieties. Following penetration of the surfactant and interaction with skin constituents, an inflammatory reaction is most probably elicited, as described earlier, involving cytokines and epidermal cells.

Increased skin permeation, required in order to achieve suitable drug concentrations in or through the skin, is not achieved without side effects, and seems to come hand in hand with increased skin irritation, as demonstrated by Kanikkannan and Singh [8]. Hence, mild skin irritation, confined to the epidermal region with structural alteration of the SC, as caused by the amphiphilogels, may be regarded as acceptable in order to achieve some degree of drug permeation through the skin barrier.

It is important to note that this study did not investigate skin sensitisation, it was particularly concerned with irritation, and not allergic reactions; however, one must remember that allergic reactions to surfactants do occur in some susceptible individuals [58,59].

4. Conclusions

The in vivo measurements of SBF in mouse, together with visual and histological evaluations of irritation, indicate that the two amphiphilogels tested, 20% w/w Span 60 in Tween 20 and 20% w/w Span 60 in Tween 80, are well-tolerated by mouse skin. The amphiphilogel tested on human skin (20% w/w Span 60 in Tween 80) was also well tolerated by volunteers when applied daily for 5 days. This is encouraging, given the high content of surfactants in the gels.

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