FISH OIL-BASED OLEOGELS: PHYSICOCHEMICALS CHARACTERISATION AND IN VITRO RELEASE OF BETAMETHASONE DIPROPIONATE

MOHAMMAD FAHMI DAMAN HURI, SHIOW-ERN NG, MOHD HANIF ZULFAKAR*
Centre for Drug Delivery Research, Faculty of Pharmacy, Universiti Kebangsaan Malaysia (UKM), 50300 Kuala Lumpur, Malaysia.
Email: hanif@pharmacy.ukm.my

ABSTRACT

Objective: The aim of this work was to develop and characterise a novel oleogel formulation using fish oil as the lipid phase for the topical delivery of BD.

Methods: Fish oil oleogels containing 0.05 % w/w BD were prepared using two types of oleogelators, namely Beeswax and Span 60. The organoleptic properties, microscopic structures, textural analysis, rheological properties, drug uniformity, storage stability and in vitro drug release profile of each oleogel formulations was investigated. A commercial preparation, Beprosone ointment was used as a control.

Results: Oleogel formulated with more than 10% w/w beeswax (FHB, FHC, FHD) displayed the most desirable properties compared to other oleogels. They showed pseudoplastic flow, smooth texture, physically stable during storage for up to six months and showed significantly higher BD flux compared to control.

Conclusion: A novel oleogel formulation based on fish oil was successfully developed and the enhancement of BD release highlighted the potential of fish oil that can be exploited as a novel vehicle for topical formulations.

Keywords: Fish oil, Betamethasone dipropionate, Oleogel, Beeswax, Span 60

INTRODUCTION

Corticosteroids remain the most commonly used topical treatment of inflammatory dermatoses including psoriasis and atopic dermatitis. The delivery of therapeutic agents through the skin is associated with challenges including variability in percutaneous absorption due to anatomical site-to-site variation [1]. Often times, high dosage of corticosteroids or very potent corticosteroids are required in order to achieve its therapeutic effects. However, this is associated with many undesirable side effects such as secondary infection, and skin atrophy with prolonged use[2].

Many studies have focused mainly on developing strategies to improve corticosteroid absorption and effectiveness of the therapy [3,4,5, 6]. An ideal topical drug for treatment of inflammatory skin diseases should be able to pass stratum corneum to reach therapeutically relevant concentrations in the epidermis or dermis without leading to high serum levels and systemic exposure [7]. Topical preparations avoid gastrointestinal irritation, prevent the metabolism of drug in the liver and increase the bioavailability of the drug [8]. Corticosteroid is a lipophilic drug hence one of the ways is to incorporate the drugs into a lipophilic delivery system.

Fish oil is one of the lipophilic bases which has a beneficial effect in enhancing the anti-inflammatory activity[9] and delivery of drugs such as betamethasone dipropionate (BD) into the skin [10]. The omega-3 and other long-chain polyunsaturated fatty acids (PUFA) contained in fish oil were reported to modulate the production of interleukin-10 (IL-10), tumour necrosis factor-α (TNF-α), interferon-γ (INF-γ) and expression of pleiotropic genes [11,12,13]. Furthermore, it is believed that the presence of these fatty acids may be an important contributing factor in the delivery enhancement [14].

Oleogels, otherwise known as lipogels, are complex microstructred systems composed of an organic hydrophobic solvent and a substance; frequently an amphiphilic molecule; able to form a three-dimensional network which immobiles the continuous medium [15]. Over the last fifteen years there has been an increase in interest in these gels and their applications were studied in many fields [16]. Recently, the main applications of oleogels are found in the field of pharmaceuticals and cosmetics [17]. In particular, successful oleogels formulations have been successfully developed with sorbitan esters as organogelators [18]. The use of wax-based vehicle is one approach to stabilize a drug which is susceptible to hydrolysis and oxidation [19].

For pharmaceutical or personal care application, oleogels must satisfy a number of criteria including long-term physical stability and rheological behaviour suitable for application, spreading and delivery of actives [20]. Furthermore, compatibility of the oil-based vehicle with the horny layer of the skin, its texture, consistency and rheological properties of plasticity make the oleogel ideal for topical administration [21].

In recent years, much attention has been focused on oil-based formulations to improve the permeability and bioavailability of poorly water soluble drug compounds. The clinical limitation of BD is its poor permeability which reduces its therapeutic effectiveness at the target site. Therefore, the major challenges for a topical formulation are to provide a sufficient increase in drug penetration into the skin [22].

In this study, our aim was to develop and characterize the physicochemical properties of fish oil oleogel formulation and determine their potential in the percutaneous delivery of betamethasone dipropionate. Two types of organogelators, namely beeswax and span 60 of various concentrations were used in oleogel formulations. The physical stability of each gel was also studied to determine the influence of different storage condition to the oleogels' rheological properties and delivery of the active ingredient.

MATERIALS AND METHODS

Materials used

Fish oil (Omega Cardiwell, Blackmores Ltd., Australia) was purchased from a local store. Analytical grade butylated hydroxiamisole (BHA) and betamethasone dipropionate (BD) were obtained from Sigma-Aldrich (M)Sdn. Bhd.(Kuala Lumpur, Malaysia). Limonene and sorbitan monostearate (Span 60) are from Merck Sdn. Bhd. (Selangor, Malaysia). Orthophosphoric acid and methanol (HPLC grade) were supplied by Fisher Scientific (M) Sdn. Bhd. (Selangor, Malaysia). Betamethasone dipropionate (Beprosone ointment) (Betamethasone dipropionate 0.064 % w/w, batch 07667005) was sourced from HOE Pharmaceuticals Sdn. Bhd. (Malaysia). All reagents were of analytical grade or equivalent, unless otherwise specified.
Preparation of oleogel formulations

Oleogel formulations were prepared by the addition of gelling agent to the oil phase and heated with constant stirring. Butylated hydroxyanisole (BHA) as anti-oxidant, beeswax and sorbitan monostearate (Span 60) as gelling agent and limonene as fragrance were also added to the oleogel formulations. Betamethasone dipropionate (BD) at a concentration of 0.05% w/w were added as the active constituent. All excipients and active ingredients as described in Table 1 were dispersed in fish oil under constant agitation and heated at 80 °C. After a clear mixture has been obtained, the solution was cooled down to room temperature in order to induce gellation.

<table>
<thead>
<tr>
<th>Formulation Code</th>
<th>Composition (% W/W)</th>
<th>Betamethasone dipropionate</th>
<th>Limonene</th>
<th>Fish oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>FHA</td>
<td>Beeswax 5 - Span 60 0.5</td>
<td>0.05</td>
<td>1</td>
<td>q.s.</td>
</tr>
<tr>
<td>FHB</td>
<td>Beeswax 10 - Span 60 0.5</td>
<td>0.05</td>
<td>1</td>
<td>q.s.</td>
</tr>
<tr>
<td>FHC</td>
<td>Beeswax 15 - Span 60 0.5</td>
<td>0.05</td>
<td>1</td>
<td>to 100 %</td>
</tr>
<tr>
<td>FHD</td>
<td>Beeswax 20 - Span 60 0.5</td>
<td>0.05</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>FHE</td>
<td>Beeswax - Span 60 5.0</td>
<td>0.05</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>FHF</td>
<td>Beeswax - Span 60 10</td>
<td>0.05</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>FHG</td>
<td>Beeswax - Span 60 15</td>
<td>0.05</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>FHH</td>
<td>Beeswax - Span 60 20</td>
<td>0.05</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Physicochemical characterization of oleogel formulations

The oleogel formulations were characterized based on organoleptic properties, microstructure observation, viscosity, pH, drug content, physical stability and in vitro drug release test. In order to establish an adequate comparison, a commercial product, Beproneson oatment was characterized in parallel.

Microstructure characterization of oleogel formulations

The microstructures of oleogel formulations were observed and photographed with polarized light microscopy (SIS 12 BIT Digital Camera Model Colour Review Olympus BX41, Japan). Each of the oleogel was spread thinly on glass slide and then observed under 40 times magnification under polarized field for the presence of crystals. The microstructures arrangement was also determined.

Mechanical characterisation

The textural analysis was performed in the compression mode in a texture analyzer (Stable Micro Systems TA-XT2i, UK), by carrying out a penetration test using cylindrical probe (5 mm diameter), a penetration depth of 10 mm and velocities of 3 mm/s. From graphic force versus time obtained, the firmness (maximum force) and adhesiveness (negative area) were calculated. The measurements were performed in triplicates.

Evaluation of rheological properties

The rheological analysis was performed with Bohlin Gemini 2 rheometer (Malvern, United Kingdom) using CP 2/20 cone plate geometry (cone diameter 20 mm, angle 2°). All formulations were subjected to pre-shear precondition to impart similar history of mechanical manipulation before apparent viscosity and oscillation rheological assay are determined [21]. This is to ensure that the internal structure of the system would break down as a result of application of a stress equal to or greater than the yield stress value for each formulation. The shear rate for precondition was applied at 200 s⁻¹ for 60 s in all samples followed by waiting time at 1 s.

Apparent viscosity

The shear applied to all of the formulations were ramped up and down at 25 °C. The shear rate ramp was applied in a logarithmic manner from 10⁻² to 10⁻¹ s⁻¹ for 198 s and 50 different points were recorded. Rheogram was obtained by plotting the applied shear rate as a function of the shear stress and the apparent viscosity was determined for each sample by using Bohlin Gemini 200 software. The rheograms were analysed to predict the flow curve pattern of each oleogel formulation.

Oscillation test

The viscoelasticity of the oleogel formulations were tested by using oscillation amplitude sweep test and frequency sweep test. In the oscillation amplitude sweep test, stress was increased from 0.1 Pa to 100 Pa. This test was applied for 217 s and 31 different points were recorded. The frequency was kept constant at 1 Hz. Three main parameters determined in this test were storage modulus (G') and loss modulus (G''), and loss tangent. The range of stress value for linear viscoelastic region (LVR) was determined and this value was used in the frequency sweep test. In oscillation frequency sweep test, the frequency was increased from 0.5 Hz to 5.0 Hz and the stress used depends on the value in the LVR.

Drug content analysis

0.1 g of each formulation was completely dispersed in 30 mg mL⁻¹ cetrimide solution to a final volume of 100 ml (0.1% w/v). The dispersion was then filtered and degassed. The drug content was analysed with high performance liquid chromatography (HPLC) according to the method outlined in below.

In vitro drug release studies

The in vitro drug release from all of the formulations and control were carried out using vertical Franz diffusion cells (Pergear, USA) and each sample aliquots are analysed by HPLC. An acetate cellulose membrane with 0.45 μm pore size was fitted between the donor and receptor compartments. The membrane was immersed in the receptor solution for 24 hours prior to the experiment. The diffusion area was 0.9 cm² and receptor chamber volume was 5.0 ml. 0.5 g of oleogel samples were spread evenly on the membrane in the donor compartment and the receptor compartment was filled with 30 mg mL⁻¹ cetrimide solution to maintain sink condition at 37 °C [23, 24]. 500 μl samples were withdrawn from the receptor compartment at 0.5, 1, 2, 3, 4, 5, 24 and 48 hours and were replaced each time with equal volumes fresh receptor solution. All samples were analysed with HPLC. The average cumulative amount of drug released per unit surface area was plotted versus time and steady state flux were determined from the slope of the linear portion of the plot. Various kinetic models such as zero order kinetic model, first order kinetic model and Higuchi were used to describe the drug release kinetics.

Determination of BD by HPLC analysis

The amount of the BD from the in vitro drug release test and drug content analysis of all the oleogels and control were determined and analysed using reversed phase high performance liquid chromatography (RP-HPLC) performed on a Waters 600E pump controller equipped with Waters 2998 photodiode array (PDA) detector and C18 column (Waters Spherisorb column, 150 mm × 4.6 mm i.d., 5 μm particle size). Runtime was 10 mins and BD was found to elute at 8.1 min. 20 μl aliquot of each samples were analysed. The mobile phase was methanol: 0.1 M orthophosphoric acid solution (70:30, v/v). Each sample were filtered and degassed prior to analysis. Limit of detection was 5 ng mL⁻¹. BD standard curve was constructed with concentrations ranging from 1 μg mL⁻¹ to 50 μg mL⁻¹ (R²=0.9992).
Physical stability of oleogel formulations

Each oleogel formulation was kept in glass and plastic containers with tight lids. Then, all of them were stored in different storage conditions which are 25 °C/ 60 % RH and 40 °C/ 75 % RH in a humidity chamber (Terchy Environmental Technology Ltd., Taiwan) and observed for 0, 3 and 6 months. For accelerated stability studies using centrifugation test, 5.0 g of each oleogel was freshly prepared and during the cool-down process, each oleogel was dispensed to 15 ml centrifugation tube. These tubes were kept at room temperature for a week to maintain their gelation properties before they were centrifuged at 2500 rpm for 30 minutes with Zentrifugen Universal 320 R centrifuge (Andreas Hettich GmbH & Co. KG, Germany).

Statistical analysis

All of the physicochemical tests were performed in triplicate and all in vitro drug release were performed with n=6. The results are expressed as mean ± standard deviation. Statistical significance was determined with one-way ANOVA test for multiple comparisons of parametric data followed by Dunnett’s posthoc test while Kruskal-Wallis test for multiple comparisons of non-parametric data followed by Dunn’s posthoc test. p < 0.05 is considered significant. The analysis was performed using IBM SPSS Statistics version 20.0 (IBM Corporation, USA).

RESULTS AND DISCUSSION

Physicochemical characterization

All of the oleogels have a citrus-like odour, which is derived from the limonene added to mask the pungent smell from fish oil. FHA and FHB both have smooth texture and good spreadability. Even though some of the oleogels have homogenous texture like FHC, FHE, FHF but they exhibited significant greasiness and stiffness. FHD, FHG and FHH showed non-homogenous properties where they have gritty textures. The greasiness, oiliness, grittiness, tackiness, stiffness or undesirable stickiness attributes uneasy feel which limit the ability of the vehicles to release medication to the skin [25]. Generally, the oleogels prepared using beeswax have good appearance compared to Span60-based oleogels, concurring with earlier studies [20, 26, 27]. Most of the oleogel showed gel behaviour but only FHE and FHF showed liquid behaviour which indicates these formulations were not as stable.

Microstructures characteristic of oleogel formulation

One of the key properties of oleogel formation is the aggregation processes of oleogel-forming materials through self-assembly and crystallization [28]. In Figure 1, all of the oleogel formulations showed formation of crystals aggregates (fat crystal network) in their microstructures where the oil phase, optically isotropic, appears dark, while crystals appear as bright white spots. The microstructures of the oleogels formed by beeswax (FHA, FHB, FHC, and FHD) are small needle-like shaped crystals known as crystal platelets (Rogers, 2009) with an average crystal length of approximately 30 µm. These crystals can be observed in Figure 1 (A), Figure 1 (B), Figure 1 (C), and Figure 1 (D). This gel is formed by arrangement of long fibres, made up of fatty acid dimers with the crystals aggregation numbers increasing in order with increasing concentration [29]. Formation of wax-based oleogels is achieved due to the presence of small microparticles of colloidal dimensions that aggregate in time to form a three-dimensional network entrapping the liquid oil [30, 31].

Figure 1 (E) and Figure 1 (F) showed the formation of small crystals aggregates in the FHE and FHF oleogel network with the absence of self-assembly structures. However, oleogel formed with high concentration of Span 60 (FHG and FHH) showed the presence of self-assembly structures which have an average tubule diameter of around 20-50 µm in their crystal network shown in the Figure 1 (G) and Figure 1 (H). These aggregates of Span 60 molecules identified by the star-shaped cluster of tubules [32] or also called as spherulites. The clusters were not a result of preformed tubules joining at a central point but rather grew as an entity [32, 33]. The manner in which gelling agent assemble and how they aggregate depends to a large extent on the gelling agent, whose component groups dictate the forces of interactions involved in gelling agent self-assembly [32].

Aggregation forces between gelling agent molecules thus include hydrogen bonding, dipole-dipole interactions, π-stacking, electron transfer, London dispersion forces, and ionic interaction, depending on the chemical structures of the gelling agent [32, 35, 36]. Moreover, the aggregation strength also depends on the intermolecular energy, which is related to the number and type of intermolecular interactions per gelling agent molecules and the number of gelling agents per aggregate thickness [16].

Fig. 1: Microstructures of oleogel formulations: (A), (B), (C), (D), (E), (F), (G), and (H) showed the microstructures of FHA, FHB, FHC, FHD, FHE, FHF, FHG, and FHH at 40x magnification. ➤ arrow represents the crystallization microstructure and ➤ represents the self assembled microstructure.
Mechanical characterization of oleogel formulations

Mechanical properties of oleogels for topical delivery are important for the maximum benefit of the patients and consumers from the formulation [39]. Texture profile analysis (TPA) defines the mechanical parameters in terms of firmness, compressibility, and adhesiveness. Texture can be regarded as a manifestation of the rheological properties of a product. Textural analysis is widely used for the mechanical characterization of semisolid products, especially in food products, cosmeceuticals, and pharmaceuticals [40].

Firmness is defined as the force necessary to attain a given deformation while adhesiveness is regarded as the negative force area calculated from AUC, where it quantifies the work required to overcome the attractive forces between the surfaces of the sample and the surface of the probe with which the sample comes into contact [41]. However, adhesiveness is a measure of the work required to remove the probe from the sample, which may in some cases involve fracture of cohesive bonds within sample, and therefore is partly dependent on sample tack [42]. Compressibility defines the work required to deform the product during the compression of probe and calculated from the positive area under the force-time curve AUC. The compressibility is correlated to the spreadability of the gel on the skin surface [43].

Table 2: Drug content percentages (n=3), steady state flux of drug released (n=6), enhancement ratio (n=6) and mechanical properties of oleogel formulations which including firmness, adhesiveness and compressibility characterization (n=3). * symbol indicates statistical significance (p < 0.05) between each oleogel formulations and beprosone control.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Firmness (N)</th>
<th>Adhesiveness (N s)</th>
<th>Compressibility (N s)</th>
<th>Drug content (%)</th>
<th>Steady state flux (μg/cm²/h)</th>
<th>Enhancement Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>FHA</td>
<td>22.98±3.904</td>
<td>-16.98±1.023*</td>
<td>64.35±12.240</td>
<td>98.73±1.608*</td>
<td>1.86±0.042 *</td>
<td>3.55*</td>
</tr>
<tr>
<td>FHB</td>
<td>59.31±5.074</td>
<td>-25.48±3.364*</td>
<td>161.52±41.243*</td>
<td>98.72±0.572</td>
<td>1.74±0.035 *</td>
<td>3.32 *</td>
</tr>
<tr>
<td>FHC</td>
<td>190.73±7.313*</td>
<td>-49.42±6.829*</td>
<td>424.32±46.305*</td>
<td>101.07±9.362</td>
<td>1.45±0.136 *</td>
<td>2.76 *</td>
</tr>
<tr>
<td>FHD</td>
<td>440.30±3.037*</td>
<td>-22.93±9.251*</td>
<td>1090.81±173.158*</td>
<td>101.65±2.244</td>
<td>0.68±0.007 *</td>
<td>1.29 *</td>
</tr>
<tr>
<td>FHE</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>99.68±0.414</td>
<td>3.16±0.108 *</td>
<td>6.02 *</td>
</tr>
<tr>
<td>FHF</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>102.29±2.608</td>
<td>2.02±0.080 *</td>
<td>3.85 *</td>
</tr>
<tr>
<td>FHH</td>
<td>15.20±1.210</td>
<td>-11.92±1.697</td>
<td>42.31±4.762</td>
<td>99.97±2.500</td>
<td>1.07±0.125 *</td>
<td>2.03 *</td>
</tr>
<tr>
<td>FHG</td>
<td>101.17±18.586</td>
<td>-91.69±25.442*</td>
<td>255.64±48.531*</td>
<td>98.75±1.275</td>
<td>0.70±0.039 *</td>
<td>1.48 *</td>
</tr>
<tr>
<td>Beprosone</td>
<td>13.55±1.572</td>
<td>-28.86±2.267</td>
<td>36.39±3.2908</td>
<td>102.82±1.663</td>
<td>0.52±0.233 *</td>
<td>1.000</td>
</tr>
</tbody>
</table>

From Figure 2 and Table 2 we determined that all of the oleogel formulations except FHA and FHG presented significantly higher firmness (F(6,35) = 577.8, p = 0.000) and compressibility (F(6,35) = 164.2, p = 0.000) compared to the control using Dunnet test for one-way ANOVA analysis. However, only FHD and FHH have significantly low adhesiveness (F(6,35) = 26.8, p = 0.000) compared to the control. In our study, we were unable to obtain the mechanical texture results of FHE and FHF because the probe of the texture analyzer had already penetrated the gels before the measurement of compressibility, firmness and adhesiveness could be taken.

The textural properties were important to determine the ease of sample removal from the container or its spreadability on mucosal or non-mucosal surface, the problems encountered during product filling and the feel of the formulation [44]. It is apparent from the results of this study that a compromise must be attained between high product compressibility, firmness and adhesiveness. In general, these parameters were increased as the gelling agent concentration increased. Compressibility and firmness quantify sample deformation under compression where they are related to the sample consistency. Products possessing high firmness and compressibility properties will be difficult to be removed from the container and applied to the skin. It is suggested that product adhesiveness is more appropriately a reliable comparative measure of affinity of candidate formulations for non-mucosal surfaces, e.g. skin [40].

Apparent viscosity of oleogel formulations

From Figure 3, the rheograms show that all of the oleogel formulations possessed plastic behaviour. This means that the oleogelshave resistance to flow at low shear rate and when the yield values are exceeded, the gels begin to thin down at high shear conditions [45]. This results in a more fluid system when subjected to external pressure and therefore spread more easily, which is a desirable characteristic for a topical gel [46].

By using Kruskal Wallis (H(6) = 25, p = 0.002), all of the formulations have significantly lower viscosity compared to the control as described in Table 3 (p < 0.05). This is due to the lower limit to yield breakdown in the network structure in the low viscous oleogels [27, 47]. The beprosone control showed very clear and high yield value, consistency and viscosity, indicating a rigid structure. The viscosity of the formulation may influence the release of drug, flow property, stability, spreadability and patient compliance [48, 49].
Amplitude sweep and frequency sweep oscillation

Rheological test parameters like storage ($G'$) and loss ($G''$) moduli, tan (δ), critical stress of linear viscoelastic region, and phase angle were obtained under dynamic conditions of non-destructive oscillatory test in the frequency range of 0.5-5.0. At the first, the LVR region for all formulations was determined by a stress sweep. The stress range over which $G'$ is independent of the applied stress is called the linear viscoelastic region (LVR). The end of linear region is called the critical stress, $σ_C$. Beyond the critical stress, the structure of the material was disturbed or breaks down. LVR value is determined by the linear range at the curve until to the critical value.

From Table 2, we determined that FHA to FHD and beprosone have larger LVR values, critical stress and $G'$ values compared to FHE to FHH. Wide LVR are signs of the ability of structures to resist external stress to a greater extent and showed more elastic behaviour materials. FHE, FHF, FHG and FHH showed a lower critical stress, $G'$ values, and LVR which indicate them less elastic behaviour materials. At stresses below the critical stress, the sample behaves like a viscoelastic solid. At higher stresses, the material starts to flow as its yield value is exceeded. In this study, Beprosone ointment showed the largest LVR value compared to the oleogel formulations. The tangent value for the beprosone control was more than 1, which indicates that the control was more viscous compared to the other.
formulations, which are considered more as elastic oleogels ($\tan \delta < 1$).

The storage modulus, $G'$, represents energy stored in the system during oscillation and is due to elastic deformation of structures in the oleogel. The loss modulus, $G''$, characterises the viscous properties. From Figure 4, the $G'$ modulus was more dominant compared to $G''$ modulus for the formulation FHA, FHB, FHC, FHD and FHH. This indicates that FHA, FHB, FHC, FHG and FHH exhibited elastic properties. Furthermore, all of them showed a gel behaviour where both the $G'$ modulus and $G''$ modulus are linear. By considering their elasticity and gel behaviour, these formulations could also be categorized as elastic solid-like gel.

The $G'$ modulus for both formulation FHD and beprosone control are higher than $G''$ modulus, which indicate that these formulations are viscous. Therefore, the formulation FHD and beprosone control showed viscous solid like gel behaviour. The $G'$ modulus for formulation FHE was slightly higher than $G''$ modulus over the frequency range up to 1.0 Hz, where a cross-over occurs. However, the $G'$ modulus started to increase and was higher than $G''$ modulus at the high frequency (> 1.0 Hz) while the $G''$ modulus showed a slightly linear profile. The formulation FHF showed almost equal $G'$ and $G''$ modulus pattern curve, which gradually increased up to 5.0 Hz. Both of the formulations showed an elastic liquid material behaviour, also known as elastic liquid-like gel.

Fig. 4: Elastic modulus ($G'$) and viscous modulus ($G''$) properties of the oleogel formulations in oscillation frequency sweep test (n=3). (A) represents viscoelastic curve for beeswax oleogel (FHA, FHB, FHC, FHD) and control while (B) represents viscoelastic curve for span 60 oleogel (FHE, FHF, FHG, FHH) and control.
Drug content analysis

From Table 2, all of the oleogel formulations have average drug content in the range of 98-102%. These showed that the drug added to the formulation during compounding process was homogenously dispersed and not degraded during preparation and that the oleogel preparation method is reproducible [48]. According to the United States Pharmacopeia (USP) Council of Experts [50], the uniformity of drug substance amount should range between 90.0% - 110.0% and a relative standard deviation (RSD) of not more than 6% as the requirement for acceptance criteria in semisolid content uniformity.

In vitro BD release from oleogel formulations

From Figure 5, all of the formulations except FHD and FHH showed significantly higher amount of BD released after 48 hours compared to the control (p<0.05) using Dunnet test for one-way ANOVA analysis (F(8,45) = 762.5, p = 0.000). The steady state flux of each formulation was determined by the slope of the curve obtained by plotting the amount of BD released per area against predetermined time interval. By using Dunn test for Kruskal Wallis analysis (H(8) = 50.9, p = 0.000), all of the formulations has a significantly higher flux than the control as described in the Table 3 (p<0.05). The enhancement ratio was determined by comparing the flux of each oleogel formulation with the flux of control formula as described in Table 2. By using Dunn test for Kruskal Wallis analysis (H(8) = 49.6, p = 0.000), we determined that all of the formulations have significantly high degree of enhancement on the release of BD across cellulose acetate membrane compared to the control (p<0.05). This showed that the fish oil as vehicle has the potential to enhance the permeation of BD from the oleogel formulation into the skin. Previous studies have also proved that fish oil can enhance the delivery of several lipophilic drugs [10, 21, 23, 51].

Fig. 5: Cumulative amount of betamethasone dipropionate released from oleogel formulations (n=6). (A) represents permeation profiles for beeswax oleogel (FHA, FHB, FHC, FHD) and control while (B) represents permeation profiles for span 60 oleogel (FHE, FHF, FHG, FHH) and control.
The mechanisms by which lipids or oils influence drug delivery, digestion and absorption are complex and not yet fully understood. Nevertheless, it is well known that lipidic excipients or hydrophobic vehicles provide a safe and effective way of enhancing bioavailability and offer an additional approach to mechanical or chemical strategies for dealing with poorly water-soluble drugs [52]. In order to understand the rate of drug release, the amount of the drug release from all of the formulations were fitted with zero, first and Higuchi kinetic model where FHB, FHD, FHG, and beprosone control followed zero order kinetic model while FHA, FHC, FHE and FHF obeyed Higuchi kinetic model (R² > 0.99).

Table 3: Rheology properties including the apparent viscosity, critical stress, elastic modulus and tan ε values in each of oleogel formulation (n=3). * symbol indicates statistical significance (p < 0.05) between each oleogel formulations and beprosone control.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Apparent viscosity at 100 s⁻¹ (Pa.s)</th>
<th>Critical stress (Pa)</th>
<th>G’ at LVR</th>
<th>Tan ε values at LVR</th>
</tr>
</thead>
<tbody>
<tr>
<td>FHA</td>
<td>0.291±0.06*</td>
<td>2.512</td>
<td>313.5±86.8</td>
<td>0.30±0.63</td>
</tr>
<tr>
<td>FHB</td>
<td>0.600±0.09*</td>
<td>3.162</td>
<td>348.5±9.0</td>
<td>0.46±0.121</td>
</tr>
<tr>
<td>FHC</td>
<td>0.725±0.046*</td>
<td>3.981</td>
<td>420.9±11.19</td>
<td>0.66±0.162</td>
</tr>
<tr>
<td>FHD</td>
<td>1.549±0.256*</td>
<td>3.162</td>
<td>1618.7±635.4</td>
<td>0.43±0.234</td>
</tr>
<tr>
<td>FHE</td>
<td>0.46±0.001 *</td>
<td>1.995</td>
<td>17.1±2.4</td>
<td>0.67±0.510</td>
</tr>
<tr>
<td>FHF</td>
<td>0.67±0.001 *</td>
<td>1.259</td>
<td>26.8±6.4</td>
<td>0.50±0.185</td>
</tr>
<tr>
<td>FHG</td>
<td>0.759±0.195*</td>
<td>0.794</td>
<td>30.4±6.8</td>
<td>0.50±0.06</td>
</tr>
<tr>
<td>FHH</td>
<td>1.359±0.109*</td>
<td>1.995</td>
<td>68.4±18.4</td>
<td>0.56±0.144</td>
</tr>
<tr>
<td>Beprosone</td>
<td>2.351±0.056</td>
<td>6.309</td>
<td>1987.7±768.7</td>
<td>1.39±0.32</td>
</tr>
</tbody>
</table>

According to the short term and long term stability studies as represented in Table 4, we determined that only FHB, FHC, and FHD were able to maintain their gel formation in both normal and accelerated condition when kept in plastic containers. Most of the oleogels packaged in glass containers were stable during storage in normal condition but the opposite in accelerated condition. FHE and FHF were unstable as they exhibited syneresis with all storage parameters. In our study, storing the oleogel in plastic container was highly preferable than glass container due to the issues observed. It has been mentioned however, efforts to achieve suitable consistency and alteration in manufacturing and packing process to meet industrial and marketing requirements do not appear to entail a practical influence on drug availability after application to the skin [27].

Storage at 40°C represents accelerated conditions that are far removed from the market conditions [20]. By taking market value considerations, it is then advisable to store the selected and stable oleogel formulations in temperatures below their phase transition temperature to avoid stability issues. However, the relevance of the modifications observed under storage at accelerated conditions could be complemented by studying the stability at an intermediate temperature [56].

Table 4: Physical stability of the oleogel formulations with different parameters and conditions: G = gel or solid, L = liquid phase (full syneresis), GL = mixture of gel phase and liquid phase (partial syneresis).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Glass container</th>
<th>Plastic container</th>
<th>Centrifugation stability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stability at 25°C/60% RH (month)</td>
<td>Stability at 40°C/75% RH (month)</td>
<td>Stability at 25°C/60% RH (month)</td>
</tr>
<tr>
<td>FHA</td>
<td>G G G G L L</td>
<td>G G G G L L</td>
<td>G G G G L L</td>
</tr>
<tr>
<td>FHE</td>
<td>L L L L L L</td>
<td>L L L L L L</td>
<td>L L L L L L</td>
</tr>
<tr>
<td>FHF</td>
<td>L L L L L L</td>
<td>L L L L L L</td>
<td>L L L L L L</td>
</tr>
</tbody>
</table>

Based on the results of the stability studies, it was clear that the stability of the oleogels is highly dependent on their composition. Previous studies reported that the oleogel developed using Span 60 were unstable for long period storage [20, 26] and the oleogels produced with beeswax represent more stable properties [26].

From the centrifugation, we determined that only FHA, FHB, FHC, FHD and FHH are stable. Centrifugation is another way to rapidly study the accelerated and stress stability compared to real time stability at 40°C. During centrifugation, the network that binds the oleogel component structure will be broken down and the internal dispersed phase (oil phase) that has a lower density will have a tendency to separate out due to the contraction of the solid network of tubules where the oil is pushed out and rise to the top of the oleogel forming a layer of oil [56].

Relationship between types and concentrations of gelling agent in oleogel formulations with the viscosity and their drug release

The types and concentrations of gelling agent can affect the release rate of a drug. In our study, we determined that the release of the BD decreased with increasing gelling agent concentration in the oleogels.
Moreover, increasing gelling agent concentrations also resulted in an increase in oleogel viscosity [59, 60] where they will form a complex and dense network structure. This would retard the movement of drug molecules within the internal structure, thus lowering release across membrane. Previous studies have discussed that most of the oleogels manufactured with beeswax above 10 % concentrations have stable ordering network structure, consistent and a high release rate [26, 27].

**Influence of microstructure polymorphism on oleogel stability**

In our study, all of the oleogel formulation showed the presence of the Maltese crosses network of microplatelet structure which indicates the formation of α-crystalline lamellar phases [61]. The presence of these Maltese crosses network and lamellar phases will increase the stability of the oleogel [62]. The instability of FHA, FHE, FFH, FHG, and FHH may be associated with a reduced number of these Maltese crosses network and lamellar phases. In FHA, FHE and FFH these decreases were shown clearly, where the number of dark areas was higher than the bright areas, indicating the presence of more liquid or oily phases than the solid gel network.

The FHG and FHF formulation instability are due to the decreased number of the Maltese crosses network and lamellar phases. In its place the presence of self-assembled tubules were increased in number. These self-assembled tubules or spherulites crystal microstructures are the result of formation of β-polymorph structures [63] where the aggregates are made up of many crystalline ribbons that grow radially from the same central nucleus [64]. The changes from the vertical α-form (platelet needle like crystal) to the tilted β-form (spherulite like crystal) occurs due to the collapse of hydrocarbon chain or bending of each molecule [65]. Similar spherulite structures also have been reported by several researchers [65, 66]. FHB, FHC, and FHD have more stable structures because they exhibit a high number of microplatelet crystal, including Maltese crosses network and lamellar phases with a more dense and compact organization [15].

**CONCLUSION**

Most of the oleogel formulations from our study have shown the ideal behaviour of oleogels, where they possess the rheological behaviour suitable for application, spreading and delivery of actives. The liquid crystals proved to be adequate systems for controlled release of active ingredient. They also showed non-Newtonian pseudoplastic behaviour, non-gritty or undesirable tackiness, high firmness and compressibility, elastic, uniformly distributed, homogenously dispersed and high release of betamethasone dipropionate compared to the commercial formulation. They also have a small, compact and dense microplatelet crystal structure which also improved their stable microstructures network and crosslink. However, we determined that only FHB, FHC, and FHD have a good long term physical stability in normal and accelerated condition with optimized plastic container. In term of drug release mechanism, the zero kinetic release proved that some of these oleogels can be maintained in the site of the application for long term drug release. This is because the repeated use of potent corticosteroid is not desirable for extended periods due to high risk of adverse effect. So, it is better if the drug release is sustained and the frequency of application is reduced. Although there are a lot of studies in developing novel dosage forms including oleogels using different techniques, process and vehicles, but there was still a lack of studies focusing on the use of fish oil. In our study, we have successfully proven that fish oil is able to enhance the delivery of lipophilic drugs and thus present a great potential for further investigations and development of fish oil as a valuable vehicle for drug delivery.

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