Development, Characterization and Ex vivo Evaluation of Various Liposome-encapsulated Aceclofenac Formulations

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Author’s contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

ABSTRACT

Aim: Formulation of aceclofenac (ACE) into liposomal gel, improving its skin permeation and potentiate its local anti-inflammatory effect.

Methodology: ACE liposomes were fabricated by lipid film hydration technique. The prepared formulations were traditional liposomes containing cholesterol (F1), ultra-deformable liposomes containing Tween 60 (F2) and modified liposomes containing both cholesterol and Tween 60 (F3). All formulations were incorporated in 1% carbopol 974 as gelling agent. The developed liposomal formulations were evaluated for its particle size, zeta potential, drug entrapment efficiency percent and stability for 90 days at 4°C. While the developed liposomal gel formulations were characterized for its in vitro ACE release and ex vivo permeation through rat skin. Anti-inflammatory effect of ACE gel formulations were evaluated in rats using carrageenan induced paw edema.

Results: The prepared liposomes had a mean vesicle size of 577 nm, 218 nm and 332 nm for F1, F2 and F3 respectively and entrapment efficiency percent of 76.8%, 58.3% and 66.2% for F1, F2 and F3 respectively. The stability study revealed that F1 vesicles have the highest physical stability followed by F3, while F2 showed the lowest stability in terms of vesicles size and entrapment.
efficiency percent. The in vitro and ex vivo results showed higher drug release and permeability from F2 and F3 than that of F1. In addition, ACE liposomal formulations have significant higher anti-inflammatory effect than that of marketed product Bristaflam cream®.

**Conclusion:** All liposomal gel formulations have the ability to enhance ACE anti-inflammatory effect in rats paw edema in comparison with marketed product Bristaflam cream®.

**Keywords:** Aceclofenac; ultra-deformable liposomes; modified liposomes; In vitro and Ex vivo evaluation.

### 1. INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) possess analgesic and anti-inflammatory effect against pain of mild to moderate intensity. They provide effective analgesia in patients after minor and major surgery [1]. While oral NSAIDs are useful in the treatment of variety of acute and chronic inflammatory diseases, their oral administration may associate with severe systemic adverse effects predominantly gastrointestinal disorders [2].

ACE is an analgesic, antipyretic, and anti-inflammatory drug and is indicated in rheumatoid arthritis, osteoarthritis, and ankylosing spondylitis. It acts on COX-2 to reduce the production of inflammation mediators [3,4]. ACE has also showed glycosaminoglycan synthesis stimulation in human osteoarthritic cartilage. It shows side effects relating to GIT, liver, and kidney functions as well as disturbance of platelets function [5,6]. It is practically water insoluble, having a molecular weight of 354.19, pKa value 4.7. It is BCS class II compound for whom oral bioavailability is decided by dissolution rate in GIT. These factors make it appropriate to formulate it in transdermal formulation [5,7].

Transdermal delivery of NSAIDs proved to be a convenient route of administration for variety of clinical indications [8]. Unfortunately, the majority of drugs do not penetrate the skin at a rate sufficiently enough for therapeutic efficacy because of the barrier function of the stratum corneum. Transdermal absorption of a medicinal substance depends on its physicochemical properties, its behavior when placed in a pharmaceutical vehicle and skin condition [9]. Therefore, pre-formulation studies are generally necessary in order to optimize drug release from the vehicle and skin permeation. There were many attempts to increase drugs flux through stratum corneum by using suitable formulation and penetration enhancers. Most of NSAIDs are lipophilic molecules which impose a big challenge in drug development and formulation to provide effective transdermal delivery systems [10].

For many years, liposomes have been used as an approach to formulate lipophilic molecules [11]. Liposomes have been shown to be a promising transdermal drug delivery system for variety of drugs especially lipophilic ones. They may act as drug penetration enhancer by increasing drug concentration in the epidermis and dermis layers [12].

Edge activators such as surfactants provide elasticity to liposomal vesicles membrane. Ultra-deformable liposomes have high deformability and could transport active agents more effectively through the stratum corneum into the deeper layers of the skin than traditional liposomes [13]. Ultra-deformable liposomes are prepared by replacing cholesterol (CH) in traditional liposomes with surface active agent.

So the aims of this work were to formulate ACE liposomal formulations in order to enhance their skin penetration, investigate the effect of Tween 60 as edge activator on liposomes (ultra-deformable liposomes) and in combination with CH (modified liposomes) on the physicochemical properties, stability, in vitro release rate and ex vivo permeability through rats’ skin after incorporating ACE liposomal formulations in 1% carbopol 974 in comparison with ACE commercial cream. As well as to evaluate the anti-inflammatory effect of ACE gel formulations employing carrageenan induced paw edema model in rats.

### 2. MATERIALS AND METHODS

#### 2.1 Materials

Aceclofenac was kindly provided by Copad Pharm Company, (Cairo, Egypt). Soybean L-α -Phosphatidylcholine (PC), Carrageenan, Triethanolamine and Cholesterol (CH) were
obtained from Sigma-Aldrich, St. Louis, MO, (USA). Chloroform, Carbopol 974 and Tween 60 was purchased from BDL laboratory Ltd., Poole, England, (UK). All solvents used in chromatographic assay were HPLC-grade and other used chemicals and solvents were commercially available products of reagent grade and used as received. Marketed formulation was Bristaflam® (containing ACE 1.5% w/w) manufactured by Smithkline Beecham, El Haram, Giza, Egypt.

2.2 Methods

2.2.1 HPLC assay method for determination of ACE concentrations

HPLC method using reverse phase adsorption chromatography was used for determination of drug concentration. The instrument consisted of a Shimadzu LC-10ATVP pump, a SIL-10AF autoinjector, an SPD-10AV UV-VIS detector, and an SCL-10A VP system controller (Shimadzu, Japan). The column was Shim-pack VP-ODS, having 4.6 mm I.D. and 150 mm bed length with adsorbent particle size 5 μm (Shimadzu, Japan). The sample was prepared in methanol and 20 μL was injected into the column. The column was eluted isocratically with Acetonitrile, methanol, and pH 7.4 phosphate buffer (20:40: 40, v/v/v) at 1 ml/min. The detection wavelength was set at 275 nm [14].

2.2.2 Preparation of ACE liposomal formulations

Lipid film hydration method was used to formulate the nanovesicles as per Table 1. The drug and lipids were accurately weighed and dissolved in a mixture of methanol and chloroform (1:2, V/V), in around bottom flask. The organic solvent system was slowly removed under reduced pressure, using a rotary evaporator (Bibby Sterilin Ltd., Stone Staffordshire, UK) at 40°C and 150 rpm, such that a thin film of dry lipids was formed on the inner surface of the flask. The dry lipid film was slowly hydrated with 10 ml of phosphate buffered saline (PBS) pH 7.4 for 1 h. The resulting liposomal suspension was mechanically shaken for 1 h using a thermostatically controlled mechanical shaker (Kottermann, Hanigsen, Germany) at 60 rpm and 40°C leading to the formation of liposomes. The liposomal suspension was left to mature overnight at 4°C, to ensure hydration of the lipid [15-17].

2.2.3 Characterization of ACE liposomal dispersion

2.2.3.1 Vesicle size and zeta potential

The mean vesicular size, size distributions expressed as the resultant polydispersity indices (PDIs) and zeta potential were determined by Zetasizer Nano series (Malvern Instruments Ltd., UK). Liposomal dispersions were diluted 10 times with phosphate buffer saline before analysis. Analysis was performed at ambient temperature. Each reported value was the average of three measurements.

2.2.3.2 Entrapment Efficiency percent (EE%)

Ultracentrifugation method was employed for determining the entrapment efficiency. An aliquot of formulation was centrifuged at 12000 rpm using ultracentrifuge (Centrifuge model no. D-7200 from Hitachi Company New Zealand) and content of drug was estimated separately in the sediment and the supernatant [18]. The entrapment efficiency was estimated as follows:

\[
\text{EE\%} = \left(\frac{T - C}{T}\right) \times 100
\]

Where T is the total drug content and C is the drug content in supernatant.

2.2.4 Physical stability of ACE liposomal dispersion

Physical stability of ACE liposomal vesicles was determined by monitoring the variations in particle size and EE% during storage at 4±0.2°C, for 3 months [15,19]. The results of stability study are shown in Figs. 1 and 2.

2.2.5 Preparation of ACE liposomal gel formulations

ACE nanovesicles were formulated into gel for ease in application. Carbopol 974 was dispersed in distilled water and dispersion (1%) was prepared. The dispersion was mechanically

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Table 1. Composition of liposomal formulations of ACE

<table>
<thead>
<tr>
<th>Formulation No.</th>
<th>Contents (mg)</th>
<th>PC CH</th>
<th>Tween 60</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1 (traditional)</td>
<td>20 - 80</td>
<td></td>
<td>80</td>
</tr>
<tr>
<td>F2 (ultra-deformable)</td>
<td>- 10</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>F3 (modified)</td>
<td>7.5 2.5 90</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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stirred for 10 min at 150 rpm using an overhead stirrer (overhead stirrer Stuart, bioCote, UK) and then neutralized by drop wise addition of triethanolamine solution (0.5%) until pH 5.5. The neutralized dispersion was kept overnight to remove any air bubble. Then nanovesicles were incorporated into the gel base and stirred for 1 h and stored at 4°C for further investigations [20]. A control formulation of marketed product Bristaflam cream® was used for comparison (F0).

2.2.6 Evaluation of the prepared ACE liposomal gel

2.2.6.1 Physical investigations

The prepared gels were tested for their color, odor, texture, phase separation or bleeding as well as the feel upon application (stiffness, grittiness, greasiness) once the preparation is applied on the skin and also after 2 min of

Fig. 1. Change in liposomes particle size upon storage at 4°C for 90 days

Fig. 2. Variation in ACE EE% upon storage at 4°C for 90 days
application. Briefly, the homogeneity of the prepared gel can be evaluated by pressing 100 mg of gel between the thumb and the index finger to notice its consistency and freedom from any coarse particles attached or detached on finger. Also, the washability can be assessed by rubbing 100 mg of gel on the skin of the back of the hand, then washed with water and observed whether it is washable or not [21]. In addition, the pH of the prepared gels (1 g of each gel formula diluted with 9 g of calibrated distilled water and mixed well) was measured by using pH meter (Jenway 3510, UK). Measurement of pH was performed in triplicates [22].

2.2.7 In vitro release of ACE from liposomal gel

Cellulose acetate dialysis membrane (Spectra Por® Dialysis membrane, Spectrom Medical Industries, Inc., USA) was utilized for the in vitro study. The membrane was soaked in phosphate buffer saline of pH 7.4 for 12 h before use. Dialysis membrane was mounted between the donor and the receptor compartments of Franz diffusion cells (Logan instruments corporation NJ, USA). The receptor compartment was filled with phosphate buffer of pH 7.4 maintained at 32±0.5°C and constantly stirred by small magnetic bar in order to ensure its homogeneity. The Franz diffusion cell had an effective permeation surface area of 1.76 cm². A known weight from each formula equivalent to 1% of ACE was added to the donor compartment. One ml of the receptor solution was withdrawn periodically over 12 h and replaced with an equivalent volume of fresh and preheated phosphate buffer saline of pH 7.4. The samples were analyzed for ACE content by the previously mentioned HPLC method against blank liposomal gels that processed similarly [14]. Experiments were carried out in triplicate and the mean of results were reported and represented in Fig. 3.

2.2.8 ACE Ex vivo permeability through rat skin

Regarding permeation study, abdominal skin of adult Albino rat weighing 192±17 g was used. Appropriate skin parts were mounted between the donor and the receptor compartments of the Franz diffusion cells with the stratum corneum facing upward into the donor compartment and the dermal side of the skin allowed to contact with constantly stirred phosphate buffer saline of pH 7.4 maintained at 32±0.5°C. A specific amount of each gel formulation equivalent to 1% of ACE was added to the donor compartment. One ml of the receptor solution were withdrawn periodically over 24 hr and replaced with an equivalent volume of phosphate buffer saline of pH 7.4, [17,23]. The samples were analyzed for ACE content. Data are shown in Fig. 4.

![Fig. 3. Dissolution profile of ACE from different gel formulations](image-url)
2.2.9 Pharmacodynamics’ evaluation of ACE gel formulations

2.2.9.1 Animals and design

Adult male Albino rats (192±17 g) were used for this experiment. The animals were maintained under standard laboratory conditions (light period of 12 h/day and temperature maintained at 25±2°C), with free access to food and water. Thirty animals were divided into five groups, six animals each. The first group (control) was received no treatment. The second group was treated with F0. The third group was treated with F1. The fourth group was treated with F2, and the last group was treated with F3. All studies were approved by the Ethical Committee of the Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt, and followed the Legislation for the protection of animals used for scientific purposes [24] and Guiding Principle in Care and Use of Animals [25].

2.2.10 Anti-inflammatory activity of ACE formulations

Carrageenan-induced hind paw edema model was used for determination of anti-inflammatory activity of ACE gel formulations [25,26]. Half an hour after dermal application of ACE formulations (1 g) on rats in each treated group (F0, F1, F2 and F3), the rats were injected with 0.1 ml of 1% W/V carrageenan solution in normal saline into the subplantar tissue of the left hind paw to produce acute inflammation.

The percent inhibition of inflammatory edema induced by carrageenan was calculated for each group using the following equation:

\[
\% \text{ of edema inhibition (anti-inflammatory activity)} = \left(1 - \frac{E_t}{E_c}\right) \times 100
\]

Where, \(E_t\) is the edema volume of treated group and \(E_c\) is the edema volume of control group at the same time point [27]. Fig. 6 shows the percent inhibition of paw edema after application of ACE gel formulations.

2.3 Statistical Analysis

All values were expressed as the mean ± standard deviation (SD) and the results were analyzed statistically by one way analysis of variance (ANOVA) followed by Tukey
Comparison Test. $P = .05$ was considered to be statistically significant.

3. RESULTS AND DISCUSSION

3.1 Preparation of ACE Liposomal Formulations

Tween 60 and CH were used in preparing liposomes for their interesting properties such as biodegradability, biocompatibility and low toxicity and derivation form easily available materials. They have been tested in various pharmaceutical drug delivery systems. All studied liposomal formulations were able to form vesicles. Liposomes appeared as translucent white dispersions that showed no sedimentation.

3.2 Vesicle Size and Zeta Potential

As shown in Table 2, the employed method in preparing ACE liposomes obtained nano-vesicles of different particle size. Incorporation of Tween 60 resulted in decreasing the particles size of the liposomes as shown in F2 ($218\pm28$ nm) while presence of CH increases liposomes particle size as in case of F1 and F3. All liposomal vesicles showed a narrow size distribution as indicated by the low values of PDIs [28,29].

Regarding the zeta potential, the surface charge of the vesicles affects the stability of liposomes [30]. Increasing the electrostatic repulsion forces between the vesicles prevents its coalescence. Zeta potential was determined after dilution of liposomes with phosphate buffer saline and the values were shown in Table 2. As was expected, presence of CH increases the rigidity of the membrane bilayer structure hence CH improves the physical stability of liposomal systems. It was reported that presence of CH in liposomes improves the stability [31,32]. In the other hand, F2 showed the lowest zeta potential value ($29.7\pm2.1$ mV) due to the absence of CH in this formulation.

3.3 Entrapment Efficiency

ACE liposomes were prepared by lipid film hydration technique. This method was proven to obtain high drug encapsulation efficiency [15,17]. The results in Table 2 indicated that, incorporation of CH in liposomal formulations improves the EE% of the formulations. The absence of CH in F2 showed the lowest EE% of value $58.3\pm4.3$. It was also reported that addition of surfactant provides deformability to the vesicles membrane and reduces drug EE% in liposomal formulations [13].

![Fig. 5. Paw edema volume (ml) after application of ACE gels in different rats’ groups compared with control one](image-url)
3.4 Physical Stability of ACE Liposomal Dispersion

At the end of storage period, all liposomal formulations showed significant changes in particle size from initial states (Fig. 1). The vesicle sizes of F1 were relatively constant at 4°C for 90 days. By the end of the storage period F1 vesicles gain 23% in size. Stability of F3 liposomes at the test conditions revealed 53% increase in vesicle size compared to the initial state. While F2 showed up to 150% increase in vesicle size which suggested high tendency to aggregate in comparison with F1 and F3. The stability study revealed that F1 vesicles showed the highest physical stability followed by F3, while F2 showed the lowest stability among the tested liposomes. The presence of CH in F1 and F3 could be the reason of the higher physical stability of these formulations.

The encapsulated ACE tended to leak out from the bilayer structure during storage. A direct relationship between the percentage leaking of the drug out of all the vesicles and aging was observed compared to initial drug EE% (Fig. 2). The drug leakage from F1 and F3 were lower than F2 at all-time points \((P = .05)\) which indicated high stability of F1 and F3. Encapsulation loss was always associated with an increase in vesicle size, which is a thermodynamically more stable status [33]. Inclusion of CH in liposomes improved the fluidity of the bilayer membrane and reduced the permeability of ACE through the membrane [31,32]. This effect lessened the leakage of the entrapped drug from the liposomes and resulted in a higher stability of the formulation.

3.5 Evaluation of the Prepared ACE Liposomal Gels

3.5.1 Physical investigations

It is obvious that all formulations have a white viscous creamy smooth texture with homogeneous appearance and ease of spreading without any separation or bleeding. All

### Table 2. Physical evaluation of ACE liposomal gel formulations

<table>
<thead>
<tr>
<th>Parameter</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particles size (nm)</td>
<td>577±39</td>
<td>218±28</td>
<td>332±33</td>
<td>-</td>
</tr>
<tr>
<td>EE%</td>
<td>76.8±5.9</td>
<td>58.3±4.3</td>
<td>66.2±5.1</td>
<td>-</td>
</tr>
<tr>
<td>PDI</td>
<td>0.219±0.18</td>
<td>0.284±0.31</td>
<td>0.273±0.37</td>
<td>-</td>
</tr>
<tr>
<td>Zeta potential (mV)</td>
<td>38.4±1.4</td>
<td>29.7±2.1</td>
<td>34.2±2.6</td>
<td>-</td>
</tr>
<tr>
<td>Lag time (h)</td>
<td>2.28±0.2</td>
<td>0.38±0.1</td>
<td>2.50±0.2</td>
<td>0.77±0.1</td>
</tr>
<tr>
<td>Permeability coefficient (cm/h)</td>
<td>5.54X10^-3</td>
<td>11.24X10^-3</td>
<td>9.56X10^-3</td>
<td>3.92X10^-3</td>
</tr>
</tbody>
</table>

Fig. 6. Percent inhibition of paw edema after application of ACE gel formulations
gel formulations have pH values in the range of 6.02–7.19 which is suitable for topical application without skin irritation.

3.6 In vitro Release of ACE from Liposomal Gels

Generally, in vitro release profile revealed important information on the structure and behavior of the formulation, possible interactions between the drug and carrier composition, and their influence on the rate and mechanism of drug release [34,35]. The dialysis release method was a well-established and useful technique to study in vitro release from micro- and nanoparticulate delivery systems. This technique has been used to study a variety of formulations, including liposomes and Nanoparticles [36].

ACE release profile demonstrated gradual and sustained release rate from the prepared liposomal formulations. The highest ACE percent released was from F2 (83% over 12 h) followed by F3 (66% over 12 h). While the lowest drug percent released was from F1 and F0. From the results it seems that formulation ingredients play a major role in determining drug release. As it mentioned before, presence of CH results in obtaining rigid bilayer membrane which hinder drug release rate. As CH content decrease, as shown in F3, or even diminished as in case of F2 drug release rate improved (Fig. 3).

3.7 ACE Ex vivo Permeability through Rat Skin

For ex vivo ACE permeation study, abdominal rat skin model was used because it is comparable to human skin in stratum corneum thickness and water permeability [37,38]. ACE permeation from liposomal gel formulations through rat skin not only affected by each formulation composition but also affected with vesicles size as well. Fig. 4 and Table 2 show the results of ACE ex vivo permeability through rat skin. Two mechanisms have been proven to enhance drug permeation from ultra-deformable liposomes. The first one is the ability to deform its shape and hence the vesicles capable of penetrating via narrow spaces delivering the drug to subcutaneous tissues [39]. The second mechanism is what so called trans-epidermal osmotic gradient. In which ultra-deformable liposomes dry over the skin due to water evaporation so the vesicles withdrawn to subcutaneous tissue rich with water content [40]. These and the smaller vesicles size explain why F2 and F3 had the higher ACE permeability through rat skin which came in agreement with other research works [40-42]. There was significant difference in permeation between F2 and F3 (P = .05), probably due to presence of Tween 60 in F2 which provide elasticity to the vesicles and hence high drug permeation. On the other hand, the relatively higher vesicles size (577±36 nm) as well as the higher CH content (20 mg) in F1 hindered and delayed ACE permeability from this formulation.

3.8 Assessment of Anti-inflammatory Activity of ACE Gel

Inflammation is a pathophysiological response of living tissue to injuries that leads to local accumulation of plasmatic fluid and blood cells [43]. Edema induced by phlogistic agents is widely accepted model for evaluation of anti-inflammatory effect of drugs. Carrageenan induced rat paw edema assay is believed to be one of the most reliable and also the most widely used test for evaluating the anti-inflammatory activity of active ingredients. Development of edema induced by carrageenan is commonly correlated with the early exudative stage of inflammation, one of the important processes of inflammatory pathology. At the beginning of carrageenan injection (phase one), there is sudden elevation of paw volume as a consequence of histamine and/or serotonin liberation from the cells [44]. After 1 h the inflammation increases gradually and is elevated after 3–6 hr. This second phase is mediated by prostaglandins, cyclooxygenase products [45].

All ACE gel formulations showed no effect on phase 1 (0.5-1 h after carrageenan injection) which was expected since ACE as NSAID has no histamine and/or serotonin inhibitory effect. On the other hand, in the biphasic process of inflammation the effect of all ACE liposomal gel formulations (F1, F2 and F3) and ACE commercial cream (F0) showed significant inhibition of edema at 1-6 h of the study in comparison to control. That could be explained by the reduction of prostaglandin synthesis via inhibiting enzymes of cyclooxygenase pathway [46]. There was significant different between all ACE liposomal gel formulations and F0 (P = .05) after one hour of carrageenan injection. This study has shown that F1, F2 and F3 enhanced ACE anti-inflammatory activity in carrageenan induced paw edema method over F0.
4. CONCLUSION

All ACE liposomal formulations have nano size particles range and high EE%. Incorporation of Tween 60 in ACE liposomal formulations decrease the particles size of the obtained vesicles and improves the in vitro and ex vivo ACE permeation while decreases physical stability of ACE liposomes. On contrary, incorporation of CH either in modified formulation or in traditional liposomes increases ACE EE% and enhances physical stability of the obtained vesicles. The prepared ACE liposomal formulations significantly improved ACE skin penetration and potentiated its anti-inflammatory effect in comparison with marketed product Bristaflam cream®.

CONSENT

It is not applicable.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

REFERENCES


