Comparative study for optimization of folic acid nanoparticles

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ABSTRACT
The present comparative study aims at studying the effect of several adjuvants on enhancing the stability and sustainability of FA NLCs which previously showed very promising results. The problem was encountered in the high drug release, high skin permeation, low depositions and low stability. In the present study, propylene glycol was added to all preparations as a stabilizer and coemulsifier. Plurol® stearique was utilized as solid lipid and stabilizer instead of Apifil®. Soft paraffin was used as a softening added to Plurol® stearique to ensure the amorphous structure of NLCs. Liquid paraffin was used for its emollient effect instead of capryol™ 90 with Apifil®. The in vitro drug release, ex vivo drug permeation and skin deposition were analyzed. Selection of the most optimum formulation achieved to investigate its photostability, long term stability. Also, it was photographed under TEM. The selected formulation was stable after six hours of irradiation. The optimized selected formulation was successfully stable in refrigerator temperature throughout 9 months of the study. The TEM photograph reveals the formation of rounded nano vesicles. The present study was successful in development of more stable, more sustained means for topical delivery of FA for future cosmeceutical benefits.

Keywords: nanoparticles; release; permeation; deposition; long term stability; photostability.

1. INTRODUCTION
Nanostructured lipid carriers (NLCs) are the second generation of solid lipid nanoparticles. They have a promising role in stabilization of the drugs [1]. NLCs are cost-effective and provide easy administration for drugs that cannot be formulated as aqueous solutions. Lipid nanoparticles appear suitable as a delivery system due to prolonged release, targeted efficiency with lower side effects and toxicity [2]. Propylene glycol is reported to act as stabilizer in preparation of NLCs [3]. Plurol® stearique is acting as a solid lipid and stabilizer and it is produced from hydrophilic and hydrophobic parts. It is suggested that the hydrophilic part facilitates emulsification and forms hard surfactant layers around the lipid nanoparticles and eventually enhance long term stability [4]. Also, NLCs prepared with Plurol® stearique are reported to possess more uniform particle size distribution during storage [5]. During storage, the particle instability may be produced by breakage of surfactant film leading to disrupted coverage of lipid surface [6]. Paraffins are used to ensure the amorphous structure of NLCs, soft appealing texture and the emollient effect desired on the skin for dermal formulations [7].

It is reported in previous studies that a final product that can be introduced to market is produced in a single step using elevated levels of lipids during the production of NLCs [8]. The lipid phase constitutes 50% of the presented formulations which possess a relatively high consistency as they are cream like and therefore, the present FA NLC formulations do not need to be incorporated in gels or creams to produce a final product easy to be applied and used. The present study suggests the possibility of production of stable sustained NLCs for the incorporation of drugs for multiple cosmeceutical benefits.

2. MATERIALS AND METHODS
2.1. Materials.
Plurol® Stearique {poly glyceryl 6- Dipalmito stearate} was a kind gift from Gattechosse, Gennevilliers, France. Propylene Glycol {99.5 %} was procured from BDH Laboratory, Poole, England. Liquid and soft paraffins were procured from El Gomhuria Company for Chemicals and Medical Trading, El-Amirya, Egypt. All other chemicals used were purchased from the same sources as the previous study reported by Ammar et al [9].

2.2. Methods.
2.2.1. Preparation.
The preparation of FA NLCs was made by hot high pressure homogenization according to Beloqui et al, 2016 [10]. The addition of propylene glycol to the aqueous phase was accomplished to all of the present optimized formulations to assist in increasing stability. F1 contains soft paraffin as an additional solid lipid to assist the amorphous structure of the NLCs. F2 contains plurol® stearique as a substitution of Apifil®. it is reported for plurol® stearique to have a stabilization effect [5]. F3 contains liquid paraffin as a substitution of capryol™ 90 to investigate its effect on release, permeation and deposition. Then the formulations were kept at -4°C for 24 h before further investigation.

2.2.2. In-vitro release study.
In-vitro release investigation was performed using a dialysis tubing non rate limiting cellulose membrane {molecular weight cut-off, 12000 - 14000 g/mol} which was purchased from Sigma-Aldrich, St. Louis, MO, USA [9]. One gram formulation was placed in the clean dialysis bag containing 10 % methanolic
phosphate buffer (pH 5.5) at 32 ± 0.5°C [11] and stirred at 100 rpm for 24 h. Five ml aliquots of the medium were sampled and replaced with fresh medium at predetermined time intervals [12]. Samples were analyzed for FA released spectro-photometrically. Experiments were conducted in duplicate. The release pattern was determined by fitting in zero, first and Higuchi kinetic models. Notice the release experiment of the previously studied FA NLCs by Hussein Ammar et al, 2016 [9] was accomplished for 6 h only.

2.2.3 Ex-vivo permeation and skin targeting studies.

The ex-vivo studies were conducted using female albino rats, 100 ± 20 g weight. The skin was excised, hair removed and extraneous fats trimmed, then washed with distilled water, examined for integrity and kept at 4°C till further use. The permeation experiments were performed using vertical Franz diffusion cells [13] with the same conditions mentioned in the paper reported by Ammar et al [9]. Half a gram of FA NLC formulations was applied onto the stratum corneum. A sample was withdrawn from the receptor medium at 1, 2, 3, 4, 5, 6 and 24 h and replenished immediately with an equal volume of methanolic phosphate buffer, pH 7.4, equilibrated at 37±0.5°C. Samples were analyzed for permeated FA spectro-photometrically [13-16]. Notice the permeation experiment of the previously studied FA NLCs by Ammar et al, 2016 [9] was accomplished for 6 h only. Triplicates were conducted.

Skin deposition investigation was conducted on the skin samples after permeation experiment by mincing skin samples, incubating in 10 ml methanol and storing in the refrigerator for 24 h. This was followed by sonication for 1 h using the bath sonicator. Afterwards, the samples were filtered by 0.2 µm syringe filters and the drug retained in skin layers was determined spectro-photometrically [17-19]. The experiment was approved by the Medical Research Ethics Committee at National Research Centre, Egypt.

2.2.4 Selection of the most optimum formulation.

Selection of the most optimum formulation was done based on lowest release, highest skin deposition and the lowest skin permeation for further stability assessments. The low release and high skin deposition help in providing more skin sustained effects.

2.2.5 Photostability of FA NLCs.

The photostability of FA NLCs was investigated according to previous reports [20, 21] by spreading a predetermined weight of the formulations on aluminum foil then subjecting them to natural sunlight from 10 am to 4 pm for 6 h. The place of accomplishment of the experiment was National Research Centre, Dokki, Giza, Egypt which is 30 m above sea level [22]. Maximum day temperature was 32 °C. At pre-determined intervals; 0, 2, 4 and 6 h; 0.5 g of each sample was withdrawn and diluted with 5 ml methanol. The quantity of drug retained was quantified using UV/VIS spectroscopy [23-25]. Also, the spectrum of free FA was plotted in order to assess the stability of free drug.

2.2.6 Long term stability study.

Stability of the selected formulation was tested by storage at refrigerator temperature (2-8°C) and room temperature (R.T.) {25°C} according to previous reports [26-28] for 9 months. Drug retained (%) as well as phase separation and particle size of FA NLCs were measured at zero time and different intervals [1, 3, 6, 9 months] [29]. Phase separation was inspected visually and particle size was tested by taking samples of FA NLCs and dilution in double distilled water in the ratio 1/1000 and short time bath sonication followed by measurement of particle size in duplicate.

2.2.7 Transmission electron microscope.

The morphology of NLC formulation {F2} was examined by transmission electron microscope with the negative stain method [30]. NLC sample was first diluted with double distilled water. A drop of the sample was applied to a copper grid coated with carbon film and air-dried, then, 2 % (w/v) phosphotungstic acid {PTA} solution was dropped onto the grids. After negative staining and air-drying at room temperature, the resultant sample was used for visualization under transmission electron microscope.

3. RESULTS

3.1 Preparation of FA NLCs.

The study by Ammar et al, 2016 [9] mentioned very promising results for F11. F11 contained Apifil® and capryol™ 90. The problem was encountered in its fast release and low stability. Three new optimized FA NLCs were successfully prepared to overcome the past problems noticed for F11.

3.2 In vitro drug release.

As shown in Figure 1; the release profiles of the present FA NLCs. The release % of FA from NLCs varies between 45.21% and 80.69% after 24 h. The release efficiency % (RE%) from NLCs varies between 30.34% and 53.63% after 24 h experiment. The maximum values are for F3 and F1 then F2. In the previous paper published by Ammar et al, the FA release % from the chosen FA NLCs reached nearly 65 % after 6 h. The results are evident for the more prolonged release of FA from our present NLCs. It is noticeable that the initial fast release (6.61% to 10.96%) is beneficial to provide rapid onset of action, which is followed by prolonged release to allow preservation of drug level via sustained release pattern [31, 32]. A biphasic release pattern is observed for FA loaded NLCs {initial burst release followed by prolonged release}. Higuchi release model is obtained (Table 1).

![Figure 1. In vitro release profiles of FA NLCs.](image)

**Table 1. Release kinetics of FA NLCs.**

| Formulation | RE [%]± SE | R² | Order of Release |
|-------------|-----------|----|-----------------|
| F1          | 46.37±2.62| 0.95| 0.78 | 0.98 | Higuchi |
| F2          | 37.08±1.68| 0.56| 0.42 | 0.74 | Higuchi |
| F3          | 53.63±6.79| 0.93| 0.79 | 0.97 | Higuchi |
It is also published that burst and prolonged release are important features for dermal delivery of drugs. Burst release enhance skin permeation of drugs and prolonged release keeps sustained therapeutic effects [8].

3.3. Permeation and skin deposition.

Figure 2 shows the skin permeation and deposition values of the present FA NLCs. The present formulations show 20.92 to 30.1 % permeation after 24 h experiment. The skin deposition values of all new optimized formulations range between 21.22% and 24.91 % after 24 h. The optimum formulation in the present study shows remarkable skin deposition value after 24 h experiment [24.91 %]. The formulations with paraffins {F1, F3} show higher permeation and lower skin deposition than formulation without paraffins {F2}. This can be refered to that paraffins have permeation promoter effect [33]. In the paper reported by Ammar et al, skin deposition value of the chosen formulation was 28.85 % after 6 h experiment and permeation value of 36.4 % after 6 h.

3.4. Selection for stability studies.

Selection of F2 is done based on the lowest release, the lowest permeation and the highest skin deposition results to assure more sustained effect.

3.5. Photostability of FA NLCs.

As shown in fig 3; it is obvious that FA encapsulated in NLCs showed excellent photostability and the drug encapsulation efficiency shows slight expulsion of the drug which doesn’t exceeds 3 % in optimized FA NLCs, F2. When FA is irradiated with ultraviolet light it is first converted to 2- amino-4-hydroxy-6-formyl pteridine {pterine-6-carboxaldehyde} and a diazotizable amine {p-aminobenzoyl-L-glutamic acid}; On further irradiation the aldehyde is converted to the corresponding 6-carboxylic acid {pterine-6-carboxylic acid} which is fluorescent and finally to the decarboxylated 2-amino-4-hydroxy pteridine [34].

Previous photostability studies evidenced that NLCs are the most efficient carriers for the preservation of drugs from UV mediated photodegradation. Nanostructured lipid carriers are reported to be able to enhance the chemical stability of compounds sensitive to light which is a very important issue concerning cosmetics and dermal pharmaceutics [35].

3.6. Long term stability study:

The physical stability of NLC formulation (F2) is monitored by particle size measurement. The mean particle size of the prepared NLCs after storage at both temperatures is shown in Table 2. The particle size ranges between 31 nm and 154.62 nm. The particle size results are not exceeding 400 nm throughout 9 months storage which implies good physical stability and is very promising for an easy penetration [36, 37]. The particle size values of F2 during this study at RT are slightly larger than in Ref T. Results show less stability, in terms of drug retained, for formulations stored at 25 °C, attributed to introduction of energy into the system leading to particle growth and subsequent aggregation and drug expulsion from NLCs [38]. The chemical stability is also evaluated by measuring the drug retained %. The percentage FA retained in F2 in room T is 83.08 % and in refrigerator T is 85.03 % (Table 2). This result implies good retention of active pharmaceutical ingredient {API} {chemical stability} which was not less than 85% in the refrigerator [39]. The absence of phase separation and homogeneity of all the present FA NLCs is evident upon storage in refrigerator (2-8°C) and room temperature (25°C) throughout the 9 months storage period.

In the previous study reported by Ammar et al [9], stability study was accomplished under the same conditions of storage; The drug loss in this previous study was below 10 % after only 3 months of storage which assure the enhanced stability of the present optimized FA NLCs.

Table 2. Percent Folic acid retained in NLCs and particle size of NLCs throughout 6 months experiment.

| Time [month] | Room Temperature | Refrigerator Temperature | FA Retained [%] ± SE | Particle size ± SE | FA Retained [%] ± SE | Particle size ± SE |
|--------------|------------------|-------------------------|---------------------|-------------------|---------------------|-------------------|
| 0            | 100              | 31.6± 8.06              | 100                 | 31.6± 8.07        |
| 1            | 87.97± 0.33      | 31.6± 14.34             | 93.56± 0.28         | 40.68± 11.12      |
| 3            | 84.65± 0.63      | 53.45± 7.4              | 90.94± 0.82         | 45.51± 5.59       |
| 6            | 83.99± 0.10      | 89.03± 4.86             | 90.93± 0.75         | 77.25± 20.01      |
| 9            | 83.08± 0.06      | 97.39± 13.94            | 85.03± 1.02         | 154.62± 28.45     |

Figure 4. Electron micrographic image of FA NLCs, F2.

NLCs were reported as physically stable for 6 months at room temperature [5, 40]. It is published in previous work that free ampicillin in an aqueous dispersion was stored for 1 month and a week at 4 °C and lost 50% of its initial activity, while by using liposome encapsulated ampicillin {free from unencapsulated drug} stored under the exact conditions lost only 17% of its initial
activity. The results of this work, give strong evidence for formulations containing ampicillin-liposome as valuable dosage forms for this drug. The mentioned study denotes that nano encapsulation of drugs is an important criterion for improving the stability of actives [39].

The present lipid nanoparticle-based systems are developed having physicochemical stability for 9 months. These bases were for proper achievement of future dermal targeting and sequential cosmeceutical benefits.

4. CONCLUSIONS

The new optimized FA NLCs, Showed more sustained release, remarkable high skin deposition and lower permeation. In addition, it was successfully stable against natural sunlight for 6 h and at refrigerator temperature throughout the period of 9 months.

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