Bull. Pharm. Sci., Assiut University, Vol. 48, Issue 1, 2025, pp. 31-48.

Bulletin of Pharmaceutical Sciences
Assiut University
Website: http://bpsa.journals.ekb.eg/

NANOEMULSION BREAKTHROUGH: A NEW MILESTONE IN ORAL DACLATASVIR DELIVERY – SCREENING, FORMULATION, *IN-VITRO* EVALUATION, AND *IN-VIVO* PHARMACOKINETIC ASSESSMENT

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The current study aimed to evaluate the efficacy of orally delivered Daclatasvir-loaded nanoemulsion (DAC-NE) for enhanced bioavailability. Solubility studies and phase diagram studies were performed to select the best formulation parameters. The formulation of DAC-NEs systems was achieved utilizing oleic acid and Tween 80/propylene glycol mix representing surfactant/cosurfactant. The key aspects of DAC-NE such as droplet size, zeta potential, pH, and transmittance were assessed. Stability was confirmed by centrifugation studies and heating-cooling cycles. The best DAC-NE system (NE-3) was selected with a droplet size of 54. 30±1.63 nm, drug content of 98.49±1.17 % and a zeta potential of -49.7±0.07 mV. Further studies were conducted for NE-3 such as morphological visualization using Transmission electron microscopy, in-vitro dissolution in addition to Fourier transform infrared analysis (FTIR), viscosity and refractive index. Results revealed complete DAC dissolution within the first 60 min from the prepared DAC-NE (NE-3), outperforming the DAC suspension and FTIR revealed compatibility between the drug and excipient components. Morphological outcomes suggest the presence of spherical globules within the nano-scale range. Furthermore, an in-vivo study using rats was conducted and revealed improved pharmacokinetic parameters of DAC from the prepared NE-3 system, where the AUC (0–∞) for NE-3 was 1.53-fold higher than DAC suspension verifying that the former recorded a notable enhancement in the level of DAC absorption and bioavailability

Keywords: *Daclatasvir, Nanoemulsion, oral delivery, in vivo pharmacokinetics, bioavailability*

INTRODUCTION

The viral infection hepatitis C virus (HCV) is a potential risk that affects plenty of citizens globally, resulting in severe complications like fibrosis, malfunctioning, hepatocellular cancer, and the potential need for liver transplantation[1-3]. At present, chronic HCV treatment has undergone a radical transformation, where an oral combination therapy of direct-acting antiviral agents has replaced the previous standard treatments such as pegylated interferon-α and ribavirin[3]. The combination therapy with several highly effective and well-tolerated drug regimens based on combined direct-acting antiviral agents therapy can improve the complications of HCV infections, including hepatosplenomegaly[4, 5].

DAC, a directly acting antiviral, has been approved in more than 60 countries for the treatment of adult HCV infection [6]. DAC significantly reduces the level of viral RNA and targets two key processes of its replication

Received : 17/9/2024 & Accepted : 2/12/2024

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by inhibiting the NS5A replication factor [7]. The commercial names of products containing daclatasvir, all administered orally, include Andodaclata, Daklinza, Clatazev, Zataciver, and Daktaviraa. Unfortunately, DAC is a class II biopharmaceutical compound [8], distinguished by its limited solubility and elevated permeability, where the solubility represents a prerequisite for the achievement of required drug levels into the bloodstream for therapeutic reply [9]. The solubility of DAC was reported to be 0.00852 mg/mL [4], resulting in low absorption and oral bioavailability of 67% [10]. A single approach has been adopted to improve the solubility of DAC, such as encapsulation of DAC into bilosomes for liver targeting and improving bioavailability[8]. NE is not a recent innovation but rather a well-established technology that has been extensively studied and refined over decades, demonstrating significant potential in enhancing drug solubility, stability, and therapeutic efficacy[11]. NE is a colloidal particulate system of submicron size [12] and boasts a distinct advantage in its minute droplet size, typically ranging from 20-200 nm[13], which greatly amplifies the interfacial surface area. Moreover, the transparent or translucent nature of NE enables its flexible administration through various pathways unlocking boundless prospects for tailoring formulations to specific needs [14].

The current study sought to establish the potential of DAC-NE orally in an attempt to develop its therapeutic efficacy. For this purpose, several oils, surfactants, and cosurfactants were screened for the best solubilizing ability for DAC. The best DAC-NE system was further evaluated using in-vitro dissolution studies in comparison with DAC suspension. In addition, the selected DAC-NE system underwent *in-vivo* trials using a rat model to assess the impact of the NE system on the bioavailability of DAC, in an attempt to achieve efficient evidence of the therapeutic spectrum to the patient.

MATERIALS AND METHODS

Materials

DAC (Marcyrl, Cairo, Egypt), acetonitrile and methanol (HPLC grade) (Fisher Co., UK), diethyl ether (SDFCL S.D. Fine Chem. Ltd. Mumbai, India), sodium acetate (Merck, Germany), potassium bromide (Sigma-Aldrich, Co., Steinheim, Germany), Rofecoxib (SIGMA Pharmaceutical Industries, Egypt), hydrochloric acid (Fluka Analytical, Steinheim, Germany), disodium hydrogen phosphate, potassium dihydrogen phosphate, oleic acid, olive oil, almond oil, sesame oil, isopropyl myristate (IPM), liquid paraffin, Tween 80, Span 80, propylene glycol, isopropyl alcohol, acetic acid solution and sodium hydroxide (El-Nasr, Cairo, Egypt).

Methods

Solubility Studies for the selection of DAC-NE components

To identify the proper NE components, solubility studies were performed on DAC where excess amounts were added to the equivalent volume (2 mL) of each vehicle (oils as oleic acid, IPM, liquid paraffin, sesame oil, surfactants as Tween 20, Tween 60, Tween 80, Span 80, and cosurfactatnts as propylene glycol, glycerin and polyethylene glycol 200) in vials and mixed for 10 min by vortex (VM-300, Gemmy Industrial Corp, Taiwan) at temperature of 37°C±0.5[15]. The vials were then agitated using a Horizontal shaker (GFL, Gesellschaft laboratories, Berlin, Germany) for 72 hrs. Subsequently, 20 min centrifugation was accomplished at rpm of 3000, and the resulting samples were passed *via* a 0.45 µm filter membrane. Post-filtration, the samples were diluted with methanol, and the DAC concentration was measured at λmax 316 nm [8] using a UV spectrophotometer (UV-1601 PC, Shimadzu, Kyoto, Japan), with the measurements performed in triplicate [13].

Construction of pseudo-ternary phase diagrams

The formulation of DAC-NE was established through an analysis of solubility studies. The key constituents employed were oleic acid, tween 80, and propylene glycol serving as oil phase, surfactant, and cosurfactant respectively. Tween 80 and propylene glycol were mixed, referred to as s/cos $_{mix}$, at various weight ratios, including 1:1, 1:2, and 2:1. To accurately determine the phase boundaries, each combination of oleic acid and the specific s/cos $_{mix}$ ratio was meticulously mixed in various weight proportions (9:1, 8:2,

7:3, 6:4, 5:5, 4:6, 3:7, 2:8 and 1:9) within glass vials. Water was then added dropwise in a controlled and gradual manner to each oil and s/cos $_{mix}$ combination, under gentle magnetic stirring (Labnet International, Inc., New Jersey, USA), until a stable and transparent system was formed. A phase diagram was constructed to represent the stable systems obtained. The phase diagram had three axes: one for the aqueous phase, another for the s/cos $_{mix}$, and a tierce for the oily phase. Each axis was distinctly denoted in the phase diagram, allowing clear visualization of the specific s/cos $_{mix}$ ratio[16, 17]. The study involved constructing phase diagrams and carefully selecting various formulations from the NE region. The focus was on varying the oil proportion while maintaining a low concentration of s/cos $_{mix}$ for the selection of formulae from the phase diagram which was then subjected to stability testing [18].

Preparation of DAC-NE systems

Briefly, DAC (60 mg) was dissolved in oleic acid, and then the appropriate amounts of s/cos $_{mix}$ were added and vortexed for about 5 min using a vortex mixer. Deionized water was gradually added, with magnetic stirring, until a uniform translucent solution became apparent[19].

Evaluation of different prepared DAC-NEs
Thermodynamic Stability

Heating–cooling cycles: Samples underwent relocation for six consecutive cycles of 4°C and 45°C (48 hours), and their stability was investigated[18].

Centrifugation study: The samples underwent centrifugation (PLC-012, Gemmy Industrial Corp, Taiwan) at 5000 rpm for 30 min. Systems that exhibited no signs of creaming, splitting, or phase segregation were chosen for subsequent analysis[17, 20].

Freezing/thawing cycles: The study was conducted on all NEs systems within a temperature range of -21°C to +25°C. Only the formulations that exhibited clarity and did not undergo phase separation were chosen for subsequent investigations[18].

Dispersibility test

The efficiency of self-emulsification for the oral DAC-NE formulae was evaluated using a standard USP XXII dissolution apparatus 2 [21] where a volume of 1mL of each DAC-NE formula was introduced into 500 mL of water maintained at 37 ± 0.5 °C [22] with paddle rotation speed of 50 rpm. The in vitro performance of the formulations was assessed visually using the following grading system[21]:

- **Grade A:** Rapidly forming nanoemulsion (within 1 minute) with a clear or bluish appearance.
- **Grade B:** Rapidly forming emulsion, slightly less clear, exhibiting a bluish-white appearance.
- **Grade C:** Fine milky emulsion that forms within 2 minutes.
- **Grade D:** Dull, grayish-white emulsion with a slightly oily appearance, emulsifying slowly (longer than 2 minutes).
- **Grade E:** Formulation demonstrating poor or minimal emulsification, with large oil globules present on the surface.

DAC-NE formulae that demonstrated successful thermodynamic stability and achieved Grade A or Grade B in the dispersibility test were selected for further studies [23].

Droplet size measurement, polydispersity index, and zeta potential

Malvern Zetasizer (MAL 104 4595, Malvern, UK) evaluated these assessments by diluting a specimen from each formula tenfold with distilled water preceding examination. PDI measurements were used to assess homogeneity concerning the size of the globules inside NE. The zeta potential (ZP) is an estimation of the DAC NE surface net charge. After processing through the Zetasizer, the electrophoresis mobility and surface-bound charges were determined [15].

pH Measurement

The pH values of all prepared DAC-NE systems were determined using a precise pH meter (Hanna-213, Portugal). The samples were carefully placed in 50 mL capacity beakers to ensure accurate measurements and the results were assessed in triplicates [13].

Percent transmittance measurement

The clarity of the formulated NE systems was assessed by conducting a percent transmittance study, which aimed to quantify the optical transparency of the samples. Briefly, 1 mL of DAC-NEs was diluted 100 times with distilled water [24]. This analysis was performed using a UV-VIS spectrophotometer, specifically measuring absorbance at 650 nm. Deionized water served as the blank control for this experiment to ensure accurate readings and minimize interference from impurities[13, 25]. The formula used to calculate the transmittance percentage, represented by "percent T," is as follows [26]:

$$A = 2 - \log \%T$$

Where: A: absorbance %T: transmittance percentage

Drug content%

For determination of the actual drug content%, 1 mL of the DAC-NEs was diluted with 10 mL methanol, and the DAC amount was determined by measuring the absorbance at λ_{max} of 316 nm against methanol as blank. Drug content% was calculated by employing the following equation [27]:

Drug content% = (Analyzed content/Theoretical content) X 100

Selection of DAC-NE

The properties of NE, as non-equilibrium systems, reflect not only the composition variables, but the processing variables such as emulsifying path, agitation, or emulsification time, which can have a considerable effect on the NE final properties [28]. The general adoption of NE can be enhanced by implementing optimization studies to acquire the optimal attributes for particular scenarios [29]. Optimization work primarily aims at exploiting the benefits offered by NE over conventional emulsions, by the attainment of small droplet size, a low polydispersity index, and a high zeta potential, respectively [30]. Consequently, based on these criteria, NE-3 was selected to represent a candidate for additional exploration.

FT-IR of the selected DAC-NE

The chosen DAC-NE (NE-3) was lyophilized complying with selection before FT-IR analysis yielded a dry powder structured article. In other words, 5 ml of NE-3 was frozen at -20 °C for 24 hours before being lyophilized with a condenser temperature of -55 °C and a vacuum pressure of 7.6 Pa at a lyophilizer (Christ Alpha 1-2LD plus, Munich, Germany) for the remaining 24 h to ensure adequate drying. Using lyophilization, the initial drying phase at -30 °C and 0.37 mbar for 12 h was followed by a further 12-h period of secondary drying at 20 °C and 0.01 mbar [31]. The FT-IR spectra of pure DAC and lyophilized NE-3 were produced by independently combining every component with KBr (IR grade) at a 2:200 ratio. The established blends were then swiftly sent to the FT-IR (IRAffinity-1, Shimadzu, Japan) for analysis at the frequency range of 4500-500 cm^{-1}.

Viscosity evaluation of the selected NE

Viscosity of the selected DAC-NE was determined using Brookfield DV III LV cone and plate rheometer (Middleboro, MA) at room temperature with spindle #3. About 40 mLs of DAC-NE sample was placed in a 100 mL glass container [32, 33].

Refractive index measurement

The refractive index of a medium quantifies the degree of interaction between electromagnetic radiation and the medium it traverses [34]. The refractive index (n) of a medium is defined as the ratio of the speed (c) of a wave, such as light or sound, in a reference medium to the phase speed (v_p) of that wave within the medium and so it could be expressed mathematically as follows [35]:

$$n = c/vp$$

The refractive index of the selected DAC-NE formula was determined using a Higler and Walt refractometer (M46.17/63707, England) where one portion of the formula was placed on the slide at 25°C [36].

***In-vitro* dissolution study of selected NE**

The *in-vitro* dissolution study of the selected DAC-NE (NE-3) formula was evaluated using the USP dissolution apparatus II. The dissolution medium consisted of 500 mL of simulated gastric fluid (SGF) with a pH of 1.2[13], prepared according to the DAC monograph in the USP. The dissolution process

was conducted at a temperature of 37 ± 0.5 ℃ and a stirring speed of 50 rpm, employing the dialysis bag technique with a molecular cut-off of 12,000 Da [8]. Accurately measured volumes of the selected DAC-NE or DAC aqueous suspension (each equivalent to 6 mg DAC) were placed in a dialysis bag which was knotted firmly at both edges and submerged in the dissolution medium. At different time intervals, 5 ml of the dissolution medium was taken to measure the concentration of DAC released, and subsequently replaced by a fresh one [37]. Aliquots were taken at 5, 10, 15, 30, 45, 60, 90, and 120-minute intervals. The concentration of DAC in each sample was measured via the results of absorbance determined at the aforementioned wavelength and the dissolution of DAC from the selected NE system was compared with that of DAC aqueous suspension [8].

Morphology examination via Transmission Electron Microscope (TEM)

The NE-3 system's morphology was investigated using TEM. A drop of the dispersed suspension was deposited on a film-coated 200-mesh copper network, stained with a drop of 2% phosphotungstic acid water solution, and allowed to dry out. The surplus liquid has been eliminated with filter paper preceding inspection. [38].

Stability studies

The selected NE system (NE-3) was securely stored in tightly sealed vials at 4 ℃ for a duration of 3 months[22]. At the culmination of the study, samples were retrieved for evaluation. The influence of storage was examined by juxtaposing the initial and post-storage observations concerning droplet size, PDI, and drug content.

***In-vivo* Pharmacokinetic Study**

Experimental Animal

The experimental protocol of the in-vivo study was endorsed by the Research Ethics Committee of the Faculty of Pharmacy, October 6 University (REC O6U) (PME-Ph-0409001). All procedures for drug administration, as well as blood and tissue collection, adhered to the guidelines outlined in the 8th edition of the Guide for the Care and Use of Laboratory Animals which was issued by the United States National Academy of Sciences in 2011. The animals were provided by the Animal House of the Faculty of Pharmacy, October 6 University (Egypt). The study used 12 male albino Wistar rats weighing between 200 and 250 grams each. The rats were housed in large, well-ventilated cages and fed regular, nutritionally balanced food.

Drug Administration and Biological Sample Collection

A total of twelve rats were randomly divided into two groups, each comprising six rats. DAC was administered at a dose of 60 mg/kg [8, 39]. The first group received oral DAC suspension in phosphate buffer (pH 6.8) [8] and the subsequent group encountered oral DAC-NE (NE-3) where both were administered *via* oral gavage, followed by a proper proportion of water. At predetermined time intervals, blood samples (1 mL) were collected *via* retro-orbital venous plexus puncture. Blood samples were gathered in heparinized vacutainer tubes and plasma was generated by centrifugation at 3500 rpm for 10 min at 4°C which was then transferred into plastic tubes and stored at -20°C.

Extraction approach and gathering the samples for analysis

Each plasma sample received 100 µL of the internal standard solution, followed by 1 minute of vortexing. Later, 8 mL of diethyl ether was added and vortexed for an additional minute. The upper organic portion was subsequently separated by centrifugation at 2500 rpm and 4°C for 10 minutes with a cooling centrifuge (model 2-16PK, Sigma). The supernatant was transferred to a clean test tube and passed through a 0.22 µm Millipore filter. The solvent was entirely evaporated when the tube had been kept in a concentrator at 60°C for 45 minutes. The residue was reconstituted with 500 µL methanol, vortexed, and filtered using syringe filters. Finally, a 20 µL sample was injected into the HPLC apparatus [40].

Chromatographic system and conditions

Plasma concentrations were quantitatively analyzed utilizing an HPLC assay method coupled with UV detection (HPLC/UV) [40]. The HPLC analysis was conducted utilizing Shimadzu's LC-20AT HPLC system, equipped

with a dual-wavelength detector (SPD-20AD Model), an automatic sampler, a quaternary pump, and a degasser. The chromatographic separation was performed on a 150 cm Inertsil® C_{18} column (4.6 mm diameter, 5 µm particle size) procured from GL Science® (Tokyo, Japan). Data acquisition and processing were facilitated through the utilization of Shimadzu's LC solution software V.1.2. A mobile phase for the chromatographic analysis was freshly formed by utilizing acetonitrile with a 10 mM sodium acetate buffer at pH 5 in an equal parts (v/v) ratio. Prior to use, the mixture was filtered and degassed employing sonication. Rofecoxib was dissolved in acetonitrile to obtain a concentration of 100 µg mL^{-1} and utilized as an internal standard [40]. The separation was carried out in isocratic mode at a flow rate of 1.0 mL min^{-1}. The effluent was monitored using a UV detector set at a wavelength of 313 nm to ensure accurate detection and quantification of the analyte [40].

Pharmacokinetic and Statistical Analysis

Non-compartmental pharmacokinetic analysis was conducted using Kinetica software (version 5.0) to compute all pharmacokinetic parameters. The area under the curve (AUC) from 0 to 24 hr (AUC_{0-24}, ng·hr/mL) of plasma concentration (Cp) versus time (t) was calculated using the trapezoidal method [8, 41].

Statistical Analysis

All experimental results were expressed as mean ± standard deviation (SD). Statistical analysis was conducted using a one-way analysis of variance (ANOVA) with SPSS® software version 22, followed by Tukey's post-hoc test, considering a p-value of less than 0.05 as statistically significant [42].

RESULTS AND DISCUSSION

Results

Solubility studies

The careful selection of primary components, including co-surfactant, surfactant, and oil, during the preparation of NEs, is crucial in formulation development [43], due to the significant impact of the drug's solubility in the NE components in maintaining the drug in a solubilized state [16]. Among the tested oils, oleic acid showed the highest solubilization for DAC (19.86 ± 1.14 mg/mL) compared to other oils (19.86 ± 1.14 mg/mL). For the surfactant and co-surfactant, Tween 80 and propylene glycol exhibited the highest capacity to solubilize DAC, consequently enhancing the transparency of the formulation., DAC displayed a notably high solubility of 30.36 ± 1.09 mg/mL and 25.63 ± 1.05 mg/m) in Tween 80, and propylene glycol, respectively (as shown in **Fig. 1**). Inclusion of Tween 80 was highly important to bring down the interfacial energy by its adsorption at the boundary of two phases, thus decreasing the interfacial tension between the oil and water. The mechanism highly favored the stability in emulsification through a reduction in the size of oil droplets and increased wetting of the drug [44]. When a surfactant alone is employed in NE formulation, achieving transient negative interfacial tension and flexible interfacial film can be challenging. However, incorporating a co-surfactant, further reduces bending stress [45]. Typically, these co-surfactants are short-chain alcohols capable of penetrating the surfactant film, enhancing interfacial fluidity, and reducing interfacial tension by occupying void spaces among surfactant molecules, thereby generating a disordered film.

Construction of pseudo-ternary phase diagrams

Based on the pseudo-ternary phase diagram provided, the phase behavior of NE systems at different surfactant-to-cosurfactant ratios was analyzed [46]. The diagram was divided into three regions based on s/cos_{mix} ratios. By examining the specific points marked on the diagram, the phase boundaries and the area of NE formed within each region can be determined [47]. The pseudo-ternary phase plot is designed so that the shaded part depicts the NE space, and the unshaded part suggests the emulsion area. The phase diagrams of oleic Acid/water/ s/cos_{mix} were constructed by Chemix Ternary Diagram software version 7.00 (Bergen, Norway) and were shown in **Fig. 2**. It is commonly anticipated that increasing the surfactant concentration enhances stability and contributes to a reduction in droplet size owing to its interfacial properties[15]. Nevertheless, exceeding the optimal level of surfactant can lead to the generation of smaller

particles exhibiting heightened Brownian motion and the onset of Ostwald ripening [48]. Alternatively, the interfacial disturbance may arise from water infiltration into oil droplets, causing their expulsion into the aqueous phase.

The phase diagram results further indicated that the dimensions and positioning of the NE zone were influenced by the proportions of surfactant and co-surfactant [49].

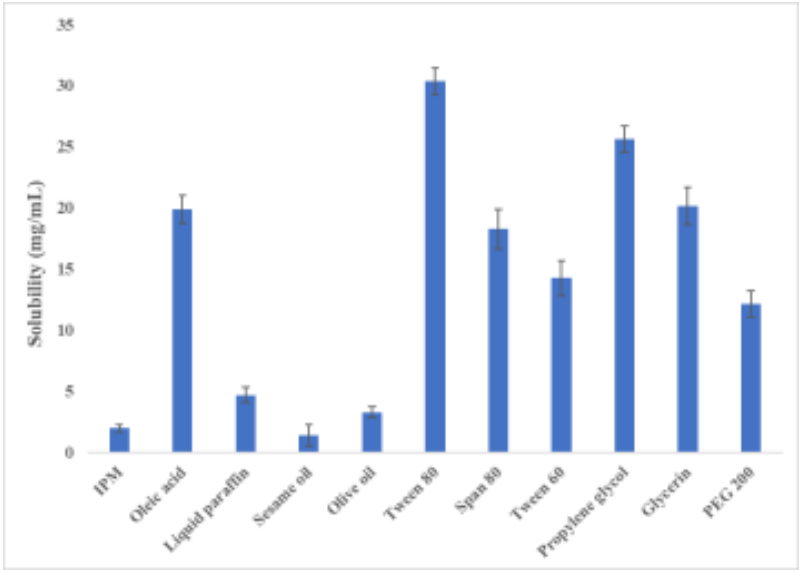

Fig. 1: Screening of DAC solubility in different oils, surfactants, and co-surfactants.

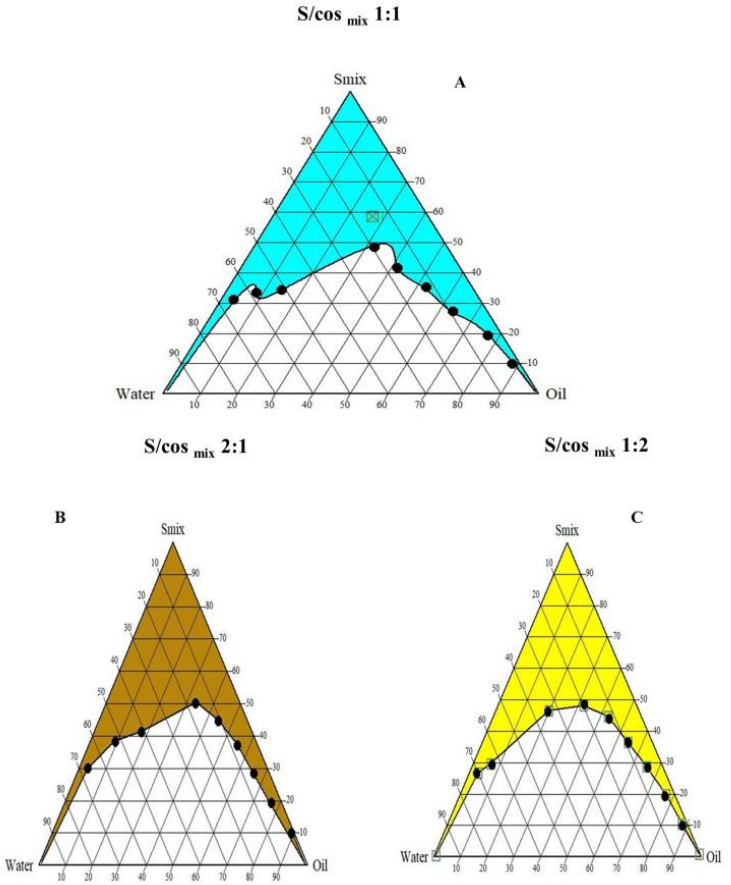

Fig. 2: Pseudo-ternary diagram of systems with different ratios of surfactant (tween 80) to co-surfactant (propylene glycol) indicating O/W NE region at different s/cos $_{mix}$ ratios. (A) s/cos $_{mix}$ ratio = 1:1; (B) s/cos $_{mix}$ ratio = 2:1; (C) s/cos $_{mix}$ ratio= 1:2.

Preparation of DAC-NE formulation

Each s/cos $_{mix}$ (Tween 80/ propylene glycol) ratio yielded six translucent liquids that flowed conveniently and had a low viscosity. In all prepared NE systems, the concentrations of oil, surfactant, and cosurfactant were systematically varied **(Table 1)**, resulting in the formation of a solubilized drug encapsulated within an oleic acid core. This core is surrounded by a protective layer of the surfactant and co-surfactant mixture, shielding the drug from the external aqueous phase [50]. No instances of drug precipitation were noted while creating NEs containing DAC. The selection of all NE systems was based on the suitability of the oil for drug solubilization and the minimization of surfactant/cosurfactant mix concentrations [51].

Evaluation of different prepared DAC-NEs

Thermodynamic Stability

All six DAC-NE systems demonstrated robust dispersion stability during testing where no indication of phase splitting or drug precipitation was monitored as displayed in **Table 2**. Consequently, they were chosen for further investigation in subsequent studies[52, 53].

Dispersibility test

When a NE formulation undergoes infinite dilution, there is a significant risk of phase separation, which may cause the precipitation of poorly soluble drugs, as NE are stabilized at specific concentrations of oil, surfactant, and water [21]. In the case of oral NE, dilution by gastrointestinal fluids leads to the gradual desorption of surfactant from the globule interface. This process is thermodynamically driven by the surfactant's need to maintain an aqueous phase concentration that corresponds to its critical micelle concentration [54]. In this study, distilled water was utilized as the dispersion medium, as previous research indicates that there is no significant difference in the characteristics of NE prepared with nonionic surfactants when dispersed in either water or simulated gastric or intestinal fluids[22,43]. The results of the dispersibility evaluation are presented in **Table 2**, where all formulated DAC-NEs are classified as Grade A or Grade B, indicating that they maintained their NE characteristics when dispersed in the gastrointestinal tract [55].

Table 1: Composition of different prepared DAC NEs.

Formula code	DAC	* S/cos $_{mix}$ ratio	Percentage (w/w) of different components in the formulation		
			Oil (oleic acid)	*S/Cosmix	Water
NE-1	60 mg	1:1	5	30	65
NE-2			10	35	55
NE-3		2:1	5	32	63
NE-4			10	38	52
NE-5		1:2	5	28	67
NE-6			10	33	57

* S/cos $_{mix}$: Tween 80/Propylene glycol.

Table 2: Thermodynamic stability, pH, drug content, and % transmittance of all prepared DAC-Nes formulae.

Formula	Thermodynamic Stability			Dispersibility Grade	pH	Drug Content	%T
	H/C	Cent	Freeze Thaw				
NE-1	Pass	Pass	Pass	A	5.68±0.25	96.16±3.08	98.95±0.18
NE-2	Pass	Pass	Pass	B	5.11±0.17	97.35±2.18	96.47±0.34
NE-3	Pass	Pass	Pass	A	5.54±0.67	98.49±1.17	99.17±0.27
NE-4	Pass	Pass	Pass	B	5.29±0.34	98.95±0.69	98.39±0.23
NE-5	Pass	Pass	Pass	A	5.45±0.41	96.72±1.24	97.92±0.13
NE-6	Pass	Pass	Pass	B	5.37±0.15	97.85±0.97	97.12±0.17

Cent: Centrifugation; H/C: Heating Cooling cycle; %T: percent transmittance
Data were expressed as mean ±S. D, n=3.

Droplet size measurement, polydispersity index, and zeta potential

The successful formulation of NE relies significantly on the accurate measurement of droplet size, PDI, and ZP, which are critical attributes [56]. The characteristics of the NE were influenced by the concentrations of oleic acid and s/cos mix. All six systems met the specified nano-scale requirements, as detailed in **Table 3** where the droplet size ranged from 54.30±1.63 nm (NE-3) to 108.41±2.15 nm (NE-6). A significant difference (p<0.05) was observed in droplet size across all prepared DAC-NE systems, potentially associated with the concentrations of oil and surfactant. The droplet size demonstrated a direct correlation with the oil concentration and an inverse correlation with the levels of surfactant and co-surfactant present. The particle size of DAC-NE correlates directly with the concentration of the oil, which serves as the dispersed phase in oil-in-water emulsions [57]. This size increase is attributed to the competition among oil particles for the limited amount of emulsifying agent present in the emulsification chamber [58]. The results also showed that droplet size decreases as surfactant concentration increases where a higher concentration of surfactants lowers surface tension and aids in globule stabilization [59]. Specifically, NE-3 system exhibited the smallest droplet size (54.30±1.63 nm). Additionally, PDI for all formulations remained below 0.4 [13, 20, 56], confirming the uniformity and homogeneity across the systems.

Zeta potential serves as a crucial indicator of NE dispersion stability, with mean values typically ranging from -27.9±0.02 to -49.7±0.07 mV, reflecting the overall stability of NE [59]. In general, zeta potential absolute values follow a guideline: values range from -5 mV to +5 mV, and aggregation develops swiftly yet values around 20 mV merely offer very short-term stability. It will be good stability if the values exceed 30 mV, but comfort in the sixth phase will demand more than 60 mV. [13]. The analysis revealed that all the prepared NE systems exhibited a notably high negative ZP charge, as illustrated in **Table 3**, signifying their stability. This elevated negative charge observed is likely attributed to the presence of anionic fatty acid groups within Tween 80[13, 60]. Maintaining a higher electrical charge on the surface of NE is essential to prevent aggregation within solutions, stemming from the strong repulsion forces between particles. This metric proves invaluable for evaluating the physical stability of colloidal dispersions, as the charge on particle surfaces, influenced by factors like surface chemistry and environment, plays a vital role in maintaining stability [16].

pH measurement

Determining the pH of oral NE is vital for ensuring safety and compliance with regulatory standards. All the produced NEs exhibited pH values within the normal oral range of 5–7 [15, 61], as evidenced by the results presented in **Table 2**, which varied from 5.11 ± 0.17 to 5.68 ± 0.25.

Percent transmittance measurement

Percentage transmittance (%T) values approaching 100% demonstrate that all DAC-NE systems are remarkably clear and transparent, allowing light to pass through with ease. This exceptional transparency is a result of their small size[13, 25], which is less than 25% of the wavelength of light. As outlined in **Table 2**, all formulated DAC-NE systems displayed transmittance percentages ranging from 96.47±0.34% to 99.17±0.27%. There is no significant difference in transmittance among all prepared DAC-NE systems (p > 0.05).

Drug Content%

The results of drug content% for all prepared DAC-NE systems ranged from 96.16±3.08% to 98.95±0.69% **(Table 2)** which falls within the acceptable range of 85–115% as specified by USP [61]. This suggests that there was neither precipitation nor loss of the drug during the formulation processes [62].

Selection of DAC-NE

The formula NE-3 was identified as the selected NE system for DAC based on its desirable characteristics: a droplet size of 54.30±1.63 nm, a low PDI of 0.107±0.01, a high ZP of -49.7±0.07 mV, and an optimal pH of 5.54±0.67. The composition of NE-3 includes 5% w/w oleic acid, 21.3% w/w Tween 80, 10.7% w/w propylene glycol, and 63% w/w deionized water.

FTIR of the selected DAC-NE

FTIR offers a method for exploring the molecular intricacies of drug interactions with NE components, facilitating the identification of specific functional groups involved in these interactions [63]. The FTIR spectra of pure DAC and selected DAC- NE (NE-3) are depicted in **Fig. 3**, where the DAC spectrum revealed characteristic bands at 3341 cm^{-1} corresponding to the N-H stretch, 2942 cm^{-1} corresponding to the C-H stretch (CH_3), 1723 cm^{-1} (C=O stretch (ester)), and 1660 cm^{-1} (C=O amide) in alignment with existing literature [64]. The presence of these peaks in the FTIR spectrum of the selected DAC NE formulation indicates compatibility between the drug and excipient components of the prepared NE [65].

Viscosity evaluation of the selected NE

The selected DAC-NE formulation demonstrated a low viscosity of 21.23 ± 2.18 cP, an essential property for ensuring pourability and ease of packaging, particularly for oral administration[66]. This viscosity falls within the typical NE range of 1-100 cP [67]. Based on these viscosity results, it can be concluded that the investigated CC-NE formulations are of the oil-in-water (o/w) type[13].

Refractive index measurement

The refractive index of the selected DAC-NE formulation (NE-3) was found to be 1.3648 ± 0.0002, which is close to the refractive index of water (1.334). This suggests that the NE formula are not only transparent, allowing light to transmit easily, but are also isotropic in nature [68]. The results fall within the acceptable range for the refractive index of NEs (should not exceed 1.476)[69, 70].

Table 3: Droplet size, polydispersity index (PDI), and zeta potential (ZP) of all prepared DAC-NEs formulae**.**

Formula code	Droplet size (nm)	PDI	ZP (mV)
NE-1	85.46±0.85	0.296±0.08	-31.6±0.04
NE-2	98.57±1.84	0.212±0.01	-27.9±0.02
NE-3	54.30±1.63	0.107±0.01	-49.7±0.07
NE-4	72.14±2.18	0.202±0.01	-39.4±0.01
NE-5	79.62±0.74	0.247±0.00	-36.5±0.03
NE-6	108.41±2.15	0.324±0.03	-42.2±0.01

Data were expressed as mean ± S.D, n=3.

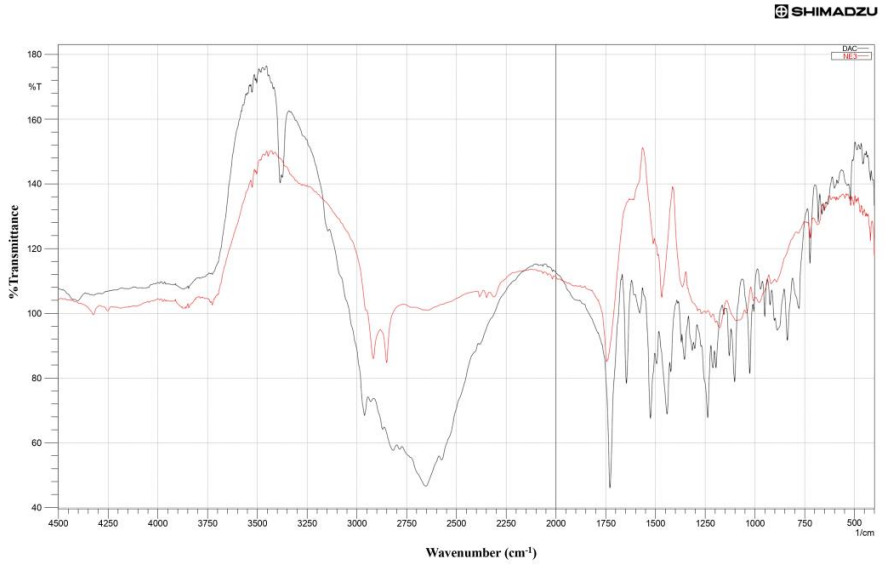

Fig. 3: FTIR spectra of pure DAC and selected DAC NE formula.

***In-vitro* dissolution study of DAC from the selected DAC-NE**

The graphical representation in **Fig. 4** depicts the comparative dissolution profiles of the selected DAC NE (NE-3) and DAC suspension. Notably, the selected DAC-NE (NE-3) succeeded in releasing 90.24±3.66% DAC after 120 min, compared to 50.69±3.14% for the DAC suspension. This could be attributed to the small droplet size, and the consequent large interfacial tension facilitates drug dissolution [61, 71], along with the drug being in a solubilized state[59]. Surfactants and co-surfactants play essential roles in NE systems by significantly lowering interfacial tension and improving the fluidity of the interface where this enhancement not only increases the mobility of the hydrocarbon tails but also promotes the penetration of oil within the NE structure [72]. These properties are particularly important when considering the *in-vitro* dissolution of drugs from NE, as the optimized interface allows for more controlled and efficient drug delivery [73]. By facilitating better interactions between the drug and the NE matrix, surfactants and co-surfactants contribute to improved drug release profiles, making them crucial for effective therapeutic applications[13].

Morphology examination *via* Transmission Electron Microscope (TEM)

The analysis revealed that the particles displayed a near-spherical morphology, specifically falling within the range of 50 nm, aligning closely with the results extrapolated from the droplet size examination. Detailed observations from the TEM images confirm that after dilution, all droplets retained a remarkably uniform spherical configuration [65], as visually evidenced in **Fig. 5**.

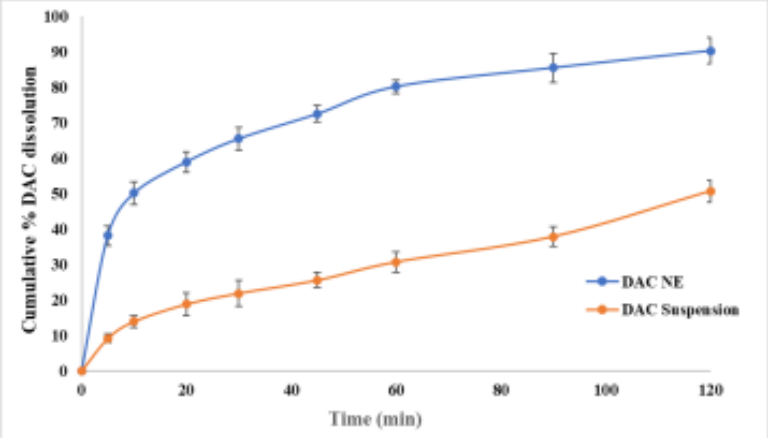

Fig. 4: *In-vitro* dissolution profile of selected DAC-NE system (NE-3) and DAC suspension in SGF (pH 1.2).

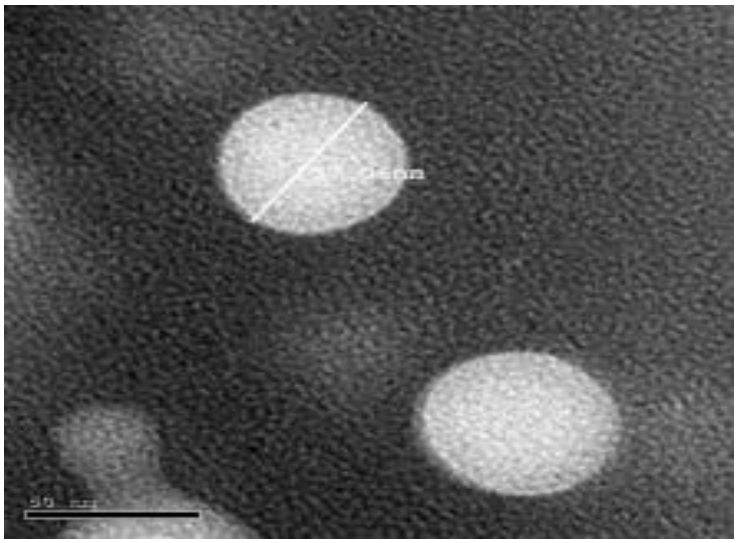

Fig. 5: Topography of selected DAC NE formula.

Stability studies

By examining the selected DAC-NE system (NE-3) after storage for three months at 4 °C, no visible changes in the physical attributes of NE-3 were observed. Moreover, the droplet size, PDI, and drug content did not show any significant differences (p>0.05) compared to the results of the freshly prepared samples. The droplet size, PDI, and drug content of the stored samples were 55.44 ± 1.28 nm, 0.105 ± 0.03, and 96.97 ± 0.27%, respectively, and those of fresh samples were 54.30 ± 1.63 nm, 0.107 ± 0.01, and 98.49 ± 1.17%, respectively. These results indicate the physical stability of NE-3 under the abovementioned storage conditions [22].

***In-vivo* Pharmacokinetic Study**

The plasma drug concentration-time curve is depicted in **Fig. 6**, with a summary of the key pharmacokinetic parameters presented in **Table 4**. A notable contrast in the pharmacokinetic profiles between the selected DAC-NE (NE-3) and DAC suspension can be observed, with a significantly higher maximum plasma concentration (C_{max}) for NE-3 (1293 ± 61.39 ng/mL) compared to that of the DAC aqueous suspension (1038 ± 54.69 ng/mL) (p < 0.05). These results confirm that the drug displayed better absorption when administered as NEs [74]. Furthermore, the AUC $_{(0-\infty)}$ value for DAC in rats receiving the DAC-NE system (NE-3) was 22463.8 ± 253.95 ng. h/mL, representing a 1.53-fold higher compared to that of the DAC suspension (14668.2 ± 148.73 ng. h/mL) (p< 0.05). Additionally, the mean residence time (MRT) of NE-3 was approximately 2 times longer than that of the DAC suspension. The enhancement in oral bioavailability may be attributed to the enhanced solubility of DAC and the more tiny the droplets, the larger their interfacial surface area, and consequently the higher the drug bioavailability[74]. The selected DAC-NE system (NE-3) produced smaller droplets with increased surface area which enabled high absorption of DAC[75]. Foraria et al. (2016) reported similar outcomes, demonstrating enhanced bioavailability facilitated by piperine NE[76]. Additionally, the study performed by Chen et al. (2017) also proved that the plasma $AUC_{0-\infty}$ of the paclitaxel NE medication is higher[77].

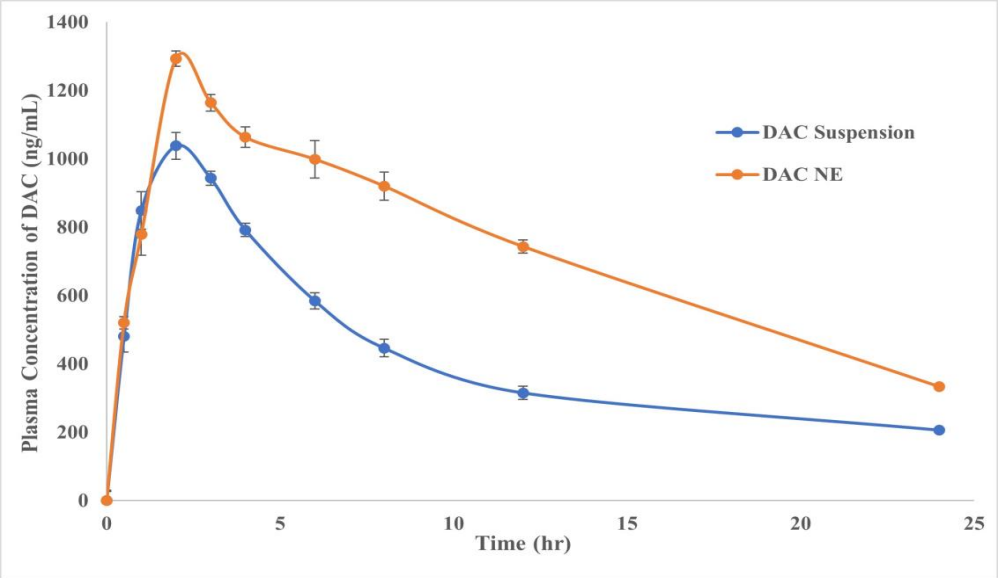

Fig. 6: Graphs showing the plasma levels of DAC in rats following oral administration of DAC suspension and DAC NE (NE-3).

Table 4 : Pharmacokinetics parameters of NE-3 (DAC NE) and DAC suspension following oral administration in rats.

Pharmacokinetic parameter	DAC NE	DAC suspension
C_{max} (ng/mL)	1293 ± 61.39	1038 ± 54.69
T_{max} (hr)	2.00 ± 0.00	2.00 ± 0.00
MRT (h)	16.79 ± 2.18	8.81 ± 1.28
AUC_{0-24} (ng.h/mL)	17251.9 ± 167.86	10228.8 ± 123.49
$AUC_{0-\infty}$ (ng.h/mL)	22463.8 ± 253.95	14668.2 ± 148.73

Data are presented as mean value ±SD; n=6.
C_{max}: maximum plasma concentration; T_{max}: time to reach maximum plasma concentration; MRT: mean residence time; AUC: area under curve.

Conclusion

The development of DAC-NE as an oral delivery system presents a real breakthrough toward increasing the efficacy and safety of HCV treatment. These DAC-NE systems were stable and distinguished by the energy of the interactions between the system's components, physical qualities, and ability to retain the drugs. Likewise, the received DAC-NE displayed faster drug release and improved drug bioavailability versus the drug suspension. The developed DAC-NE has the potential to tackle worldwide chronic HCV infections by improving solubility, stability, and therapeutic efficacy. The enhancement in oral bioavailability of DAC through NE formulation does provide hope for application in the clinical setting. The results open a pathway for further research studies and clinical trials that might eventually result in the development of an innovative and potent oral treatment for Hepatitis C infection patients.

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Bull. Pharm. Sci., Assiut University, Vol. 48, Issue 1, 2025, pp. 31-48.

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انجاز المستحلب الهلامي النانو: طرق جديدة في توصيل Daclatasvir عن طريق الفم – الفحص والصياغة والتقييم في المختبر وتقييم الحركية الدوائية في الجسم الحي

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هدفت الدراسة إلى تقييم فعالية المستحلب الهلامي النانو المحمل Daclatasvir (DAC-NE) الذي يتم تناوله عن طريق الفم لتحسين التوافر البيولوجي. أجريت اختبارات مختلفة لتحسين معلمات الصياغة، بما في ذلك دراسات الذوبان. تمت صياغة DAC-NEs باستخدام مكونات محددة. تم تحديد نظام DAC-NE الأفضل أداء (NE-3). تم إجراء تحليلات إضافية، بما في ذلك التصور الشكلي ودراسات الذوبان في المعمل. أظهرت النتائج انحلالا سريعا ل DAC مع NE-3 ، مما يشير إلى تعزيز الاتاحة الحيوية. أظهرت دراسات الفئران في الجسم الحي تحسين عوامل حركية الدواء في صورة المستحلب الهلامي النانو، مما يؤكد امتصاصا أفضل وتوافرا للدواء مع نظام المستحلب الهلامي النانو.