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Preparation, Evaluation, and Histopathological Studies of Ondansetron-Loaded Invasomes Transdermal Gel

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ABSTRACT: Ondansetron is a serotonin receptor antagonist for treating nausea and vomiting. Its multiple daily doses and extensive first-pass metabolism faced parenteral and oral administration challenges. The transdermal route enhances its bioavailability as it bypasses the first-pass effect. Invasome vesicles improve the skin permeation of ondansetron via its unique components, lecithin, limonene, and ethanol, which are considered efficient permeation enhancers. The current study intends to develop a gel containing ondansetron as invasomes vesicles with appropriate texture and efficient skin penetration. Two gelling agents, hydroxypropyl methylcellulose K_4M and carbopol 934P, were used at two concentrations (0.5 and 1%, w/w). Formulas were evaluated regarding pH, content uniformity, viscosity, spreadability, and ex vivo permeation. The selected formula (F3), which contained ondansetron (5%, w/w) and used carbopol 934P at (0.5%, w/w), was homogeneous, with proper viscosity (21,500 ± 390 mPa.s) at rest and enough spreadability (3.4 ± 0.45 cm). The ex vivo permeation showed permeation flux (Jss) was (280.4 ± 3.5 g/cm2.h) with a shorter lag time (0.5 ± 0.1 h). Characterization studies revealed nanosized vesicles (Dav. = 202.9 ± 0.5 nm) as spherical shapes dispersed within a gel matrix. In vivo, skin irritation and histopathology studies using male Wistar albino rats demonstrated that (F3) was biocompatible, with no irritation reported for six consecutive days. In conclusion, the invasomes gel of ondansetron utilizing carbopol (0.5%, w/w) was prepared for transdermal delivery, offering effective permeation and safe application. An invasomes-loaded gel is considered a novel formulation, giving an efficient and realistic therapeutic option for treating vomiting.

KEYWORDS: Invasomes; Ondansetron; Carbopol 934P; HPMC K_4M; Limonene; in vivo skin irritation study.

1. INTRODUCTION

The oral route is the most common non-invasive route for drug administration. However, most of the therapeutic activity of drugs was lowered via the oral route. The extensive first-pass metabolism is one of the reasons for lowering the oral bioavailability of drugs before eliciting their systemic action [1].

On the other hand, multiple daily doses of drugs with short action are needed for oral and parenteral routes. The transdermal delivery system bypasses the first-pass effect, increasing the amount of drug available that enters the systemic circulation, offering a non-invasive route, and improving patient compliance, especially for patients unable to administer the drug orally or parenterally [2].

Transdermal drug candidates have a short half-life (< 10 h), are lipophilic (log K(o/w) = 1 – 4), have a low melting point (200 – 250 °C), a low molecular weight (< 500 g/mol) and low dose (< 20 mg/day) [3].

Invasomes are newly developed vesicles made from phospholipids (e.g., egg lecithin and soybean lecithin), ethanol, and terpenes. Terpenes are volatile oils of either monoterpenes or diterpenes type (cineol, fenchone, and limonene) with a low boiling point which they are used for many years in dermal applications and cosmetics and are deemed safe for human consumption [4].

Ondansetron (ONDS), a serotonin receptor (5 - HT3) antagonist, treats nausea and vomiting induced by illness, surgery, chemotherapy, radiotherapy, and pregnancy [5]. Ondansetron is a weak base with a pKa of 7.4 (an amphiphilic molecule); its salt is soluble in acidic media and considered a class-II drug (low soluble, high permeable) according to the biopharmaceutics classification system (BCS) [6]. The logP is (2.07), melting point (230 – 235 °C), and molecular weight (293 g/mol) make it a good candidate for transdermal delivery. After oral administration, ONDS suffers from an extensive first-pass effect, contributing to its low oral bioavailability (50 - 60%). On the other hand, ONDS t1/2 is 3 –ıı 4 h, so frequent doses are needed to achieve the therapeutic effect. These considerations urge researchers in the field of pharmaceutical formulations to identify a suitable alternative route for ondansetron administration [7].

Previous studies showed that the permeation of ONDS across rat skin was enhanced using nanoparticles technology [8]. In addition, studies have shown that, when formulated as liposomal dispersion, results showed that liposomes have more efficient penetration across rat skin than ONDS solution [9].

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Gelling agents like carbomer and cellulose derivatives are polymers commonly used to prepare gel formulations. Carbopol 934P (CA) is a synthetic acrylic polymer that is non-toxic, biocompatible, and nonexpensive. On the other hand, cellulose derivatives polymers are another non-toxic compound obtained chemically from methylcellulose and hydroxypropyl ether. Various products, such as the E, F, and K series, have been utilized in various applications [10]. Like hydroxypropyl methylcellulose (HPMC K_4M), the K series is a hydrophilic gel-forming matrix for sustained release preparations [11]. The choice of the gelling agent depends on many factors, e.g., physical properties, route of administration, and suitability [12].

The current study attempts to design a formulation of ONDS that is an alternative to the existing oral and parenteral routes by preparing and developing ONDS invasome dispersion as a transdermal gel. It proposes enhancing the permeation of ONDS invasomes across skin layers and identifying the safety of the prepared formulations upon application on the skin. The study hypothesized that invasomes ensure efficient permeation of ONDS and subsequent release within skin layers and systemic circulation to elicit its antemetic action systemically. The study seeks to select the optimum gelling agent with accepted physical and chemical properties that efficiently release, permeate, and have minimal skin retention and safe skin application of ONDS-loaded invasome vesicles.

2. RESULTS AND DISCUSSION

2.1. Formula's physical appearance

Blank gels appeared clear and transparent, while the color of the prepared formulas appeared white to yellowish. Results showed that all formulas were homogeneous with a smooth texture, no odor, and free from grittiness. Phase separation was observed in (F1 and F2), which use HPMC K_4M as a gelling agent after 2 months of storage at (25 ± 3 °C), while no phase separation in (F3 and F4) was reported, which might be related to the stability issues of the cellulose derivative polymer, that was more prone to be affected by storage factors. Carbopol 934P showed a more stable structure with time, favoring its use more frequently than cellulose derivative polymers [13]. All formulas were washable with water, neutral, and compatible with skin pH (pHskin = 7.2 - 7.4). The pH values ranged from 6.8 ± 0.2 for F1 to 7.7 ± 0.4 for F4, suggesting no significant difference ($p > 0.05$) among formulas [14].

2.2. Content uniformity

Formulas (F1 to F4) have (102.2 ± 1.7%, 99.4 ± 1.2%, 100.5 ± 2.9%, and 98.1 ± 2.1%, w/v) ONDS content, respectively. Accordingly, all formulas are considered acceptable according to the United States Pharmacopeia (USP) stipulates that the uniformity of the first ten dosage units should fall within the range of (100 ± 15%) for accepted content uniformity [15].

2.3. Viscosity

The data obtained demonstrated reduced viscosity values with increasing rotation speed (Rpm), resulting in pseudoplastic flow with thixotropic properties [16]. The viscosity values of blank gels at 20 rpm (at rest) were (2322 ± 113 mPa.s and 14740 ± 188 mPa.s) for HPMC K_4M and CA, respectively. Results revealed that (F1 and F2) have significantly lower ($p < 0.05$) viscosity values (3620 ± 110 mPa.s and 6932 M ± 106 mPa.s) at 20 rpm, (1180 ± 89 mPa.s and 2438 ± 40 mPa.s) at 200 rpm, compared to (F3 and F4) which has (21500 ± 385 mPa.s and 39560± 330 mPa.s) at 20 rpm and (6410 ± 153 mPa.s and 11610 ± 210 mPa.s) at 200 rpm. With the considerable molecular weight of acrylic acid crosslinked with allyl ethers of poly alcohols, CA offers sufficient viscosity and retention at the application site. In addition, the crosslinked framework increases the consistency at neutral pH [17]. Furthermore, the effect of gelling agent concentration showed that increasing the concentration of CA from (0.5 to 1%, w/w) had a significant effect ($p < 0.05$) on viscosity, as shown in (F3 and F4) while increasing the concentration of HPMC K_4M had no significant effect ($p > 0.05$) on viscosity values as in (F1 and F2) [18].

2.4. Spreadability

Spreadability is a physical property of effortlessly applying the formulation on the skin. A formula that spreads quickly with simple rubbing was preferred [19]. Results showed (F1 and F2) containing HPMC K_4M have significant spreadability distance ($p < 0.05$) (5.1 ± 0.76 cm and 3.8 ± 0.76 cm) when compared to (F3 and F4) containing CA (3.4 ± 0.45 cm and 1.9 ± 0.15 cm). The effect of increasing gelling agent concentration from (0.5 - 1%, w/w) demonstrated no significant differences ($p > 0.05$) in spreadability distance for (F1 and F2). In contrast, results showed a significant decrease ($p < 0.05$) in spreadability distance upon increased CA concentration for (F3 and F4). Viscosity is inversely proportional to spreadability. Spreadability was related to

the viscosity of the gelling agent; as HPMC K_4M is less viscous than CA, the spreadability distance is higher for HPMC K_4M than CA [20].

2.5. The ex vivo permeation

The HPLC analysis method developed was valid, accurate, reproducible, and sensitive for determining ONDS [21]. It was separated as a single clear peak at (3 ±0.4 min) retention time. Six concentrations, including the lower limit of quantitation (LLOQ), were used to validate the linearity of the analysis method. The (LLOQ) is the lowest concentration, equal to (2 ± 0.3 µg/ml). The limit of detection (LOD) of ONDS was (0.65 ± 0.1 µg/ml). The linearity was achieved as the correlation coefficient (R2) reached unity (R2= 0.99). The average percent recovery of ONDS was (98.25 ± 0.48%). The results of permeation flux (Jss) (Figure 1) were (243.13 ± 5.2, 216.94 ± 4.1, 280.4 ± 3.5, and 203.5 ± 5.8 µg/cm2.h) for (F1, F2, F3, and F4), respectively. After 9 h permeation time, all formulations exhibit a substantial permeation rate owing to the unique content of invasomes (limonene, lecithin, and ethanol) regarded as effective permeation enhancers. Limonene's high lipophilicity (logP= 4.83) and lower boiling point (176 °C) compared to other volatile oils are two of its most notable characteristics that facilitate efficient association of invasomes with the epidermal layers and remarkably enhance ONDS permeation across skin layers [22]. On the other hand, ethanol might disturb the skin's stratum corneum (SC) layer, reduce tight junctions, and increase skin permeability. Several potential mechanisms, including lipid extraction from the lipid bilayer matrix, fluidization of the lipid bilayer, modification of SC protein conformation, co-permeation of drug with ethanol (pull effect), and improvement of drug solubility in the SC lipids, were derived from earlier investigations [23]. There was no statistically significant difference ($p> 0.05$) in (Jss) among formulations, and all were close to the desired flux value. Therefore, it was noticed that the type of gelling agent did not impact the penetration of ONDS through skin layers. Additionally, the permeation time was significantly affected ($p< 0.05$) by increasing the gelling agent concentration from (0.5 - 1%, w/w) as in (F2 and F4). As the gelling agent concentration increased, the gel's viscosity increased, and the polymer matrix's erosion time extended. This behavior was observed when the gelling agent was in contact with the buffer medium of the receptor compartment of the Franz cell. The gelling agents used were hydrophilic, and as a result, the gel swelled, and a viscous, gelatinous layer formed on the matrix's outer surface. This layer controls the release of ONDS from the matrix, in which increasing the gel's viscosity increases its resistance to erosion and ONDS diffusion, resulting in a slower rate of ONDS release [24]. Hence, an extended time of ONDS from invasomes was noticed in which (F2) released 3.25 ±0.05 mg of ONDS and (F4) released 3.15 ± 0.08 mg after 9 h, respectively. While (F1) released 3.7 ± 0.03 mg and (F3) released 4 ±0.05 mg of ONDS after 9 h, respectively. Choosing the formula that released more ONDS during the study is reasonable. As noticed for all formulations, the release and permeation across the skin membrane was an extended type, and no additional merits were obtained when the release and permeation were slower. Hence, (F3) was satisfied for achieving release and permeation time with more ONDS than other formulas. Lag time (T lag) was considerably lengthened ($p< 0.05$) for (F2 and F4) (1.2 ± 0.8 h and 1.35 ± 0.1 h), respectively, as the concentration of the gelling agent increased. Compared to (F1 and F3), they had a shorter lag time (0.75 ± 0.4 h and 0.5 ± 0.1 h), respectively. Lag time is related primarily to the consistency of the gelling agent that might hinder the release of ONDS vesicles from the gel matrix [25].

Equation 1 estimated the area of application for human skin required to obtain (Css) and provide a maximum therapeutic effect [26].

(Eq.1) $\text{Area} = (\text{Daily transdermal dose} \times 3)/(t \times Jss)$

Where, (t) = 24 h, (Jss) is the observed flux in an ex vivo study which is equal to (280.4 ± 3.5 µg/cm2.h) for (F3), and (3) is the correction factor for prediction of flux in humans based on the ex vivo study. The reported total daily oral dose of ONDS is (24 mg). As the oral bioavailability of ONDS is 60%, the transdermal dose equals (14.5 mg), and the area calculated is (6.5 cm2). Therefore, the predicted transdermal dose of ONDS invasomes gel is 45.5 mg (≈ 50 mg), which corresponds to (1 g) invasomes gel of (5%, w/w). Despite the oral dose being (14.5 mg), the transdermal administration depends on the area of application from which ONDS is determined, ensuring a high thermodynamic activity of ONDS in the gel reservoir [26].

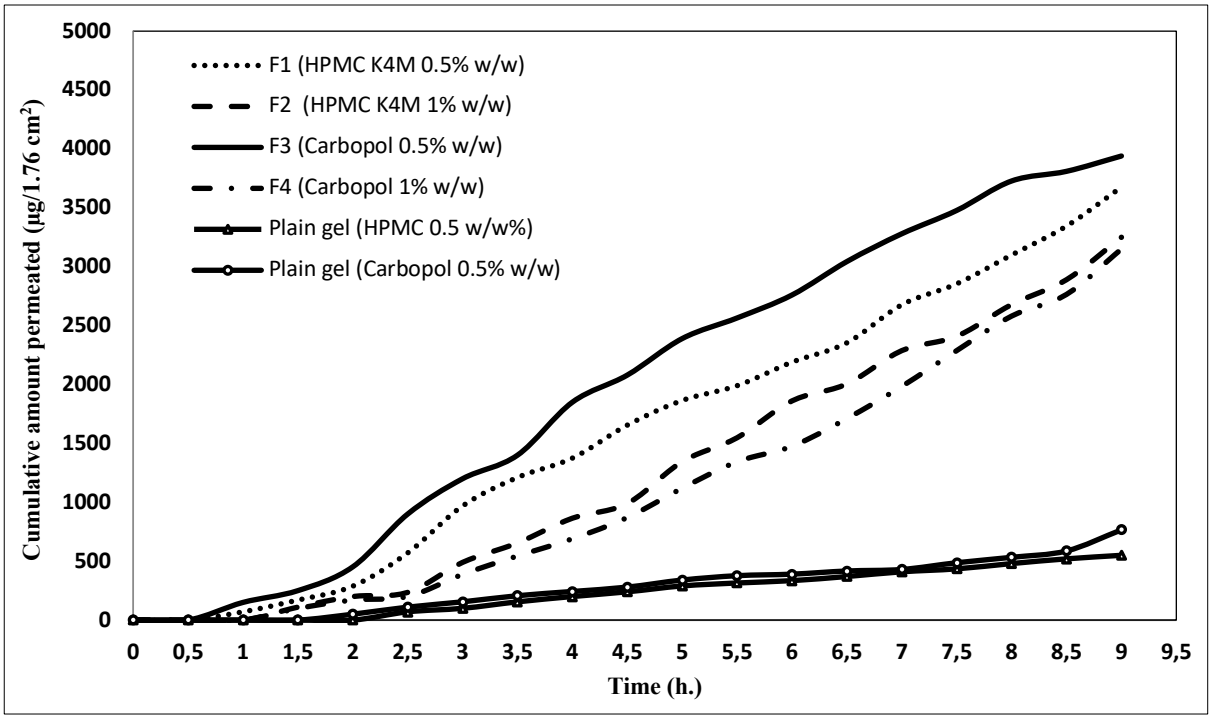

Figure 1: The ex vivo permeation for the prepared formulas across rat skin (1.76 cm2 surface area) at (32 ±1 °C). Error bars (I) represent standard deviation (SD), (n=3)

The selected formula (F3) was considered for further study. It exhibited no phase separation during storage and was homogenous. In addition, it had sufficient viscosity (21550 ± 385 mPa.s)to be retained and adhere to the skin at rest, sufficient spreadability (3.4 ± 0.45 cm), effective permeation (Jss) (280.4 ± 1.5 µg/cm2.h), higher amount of drug permeated ≈ 4 mg after 9 h and a shortened (T lag) (0.5 ± 0.1 h). In addition, (F3) demonstrated a six-fold increase in (Jss) (280.4 ± 3.5 µg/cm2.h) compared to the plain gel (44.88 ± 1.7 µg/cm2.h).

2.6. Compatibility results

2.6.1. FTIR

The FTIR spectrum of ONDS shown in (Figure 2-A) demonstrates the positions of the characteristic peaks of the ONDS molecule. The results agreed with the references, indicating the drug's identity. A broad shape band (3372 cm-1) related to N-H stretching was reported. In addition, a sharp stretching band at (2932 cm-1) for C-C aromatic and a sharp band of C=O stretching (N-H bending) at (1620 cm-1) (Amide I) were recorded. In addition, other bands at (1455 cm-1) for C≡N stretching (Amide II) and (753 cm-1) for C-H bending were also examined [8]. Results in (Figure 2-B) show that CA had characteristic bands at (3128 cm-1) for OH stretching and a sharp peak at (1701 cm -1) for C=O stretching. The ONDS: CA physical mixture (Figure 2-C) shows more broadening in the N-H stretching in ONDS when incorporated in CA, suggesting H-bond formation. The presence of broadening in bands at (3309 cm-1 and 1635 cm-1) in (F3), as in (Figure 2-D), shows that the position of the N-H, C-C, and C=O stretching bands in ONDS had not been changed, and no incompatibilities have been reported. However, the transmittance of the bands significantly lowered when compared to the ONDS spectrum or ONDS: CA due to the entrapment of the ONDS in invasome vesicles that dispersed in CA gel, which led to an increase in the intensity of the bands [27].

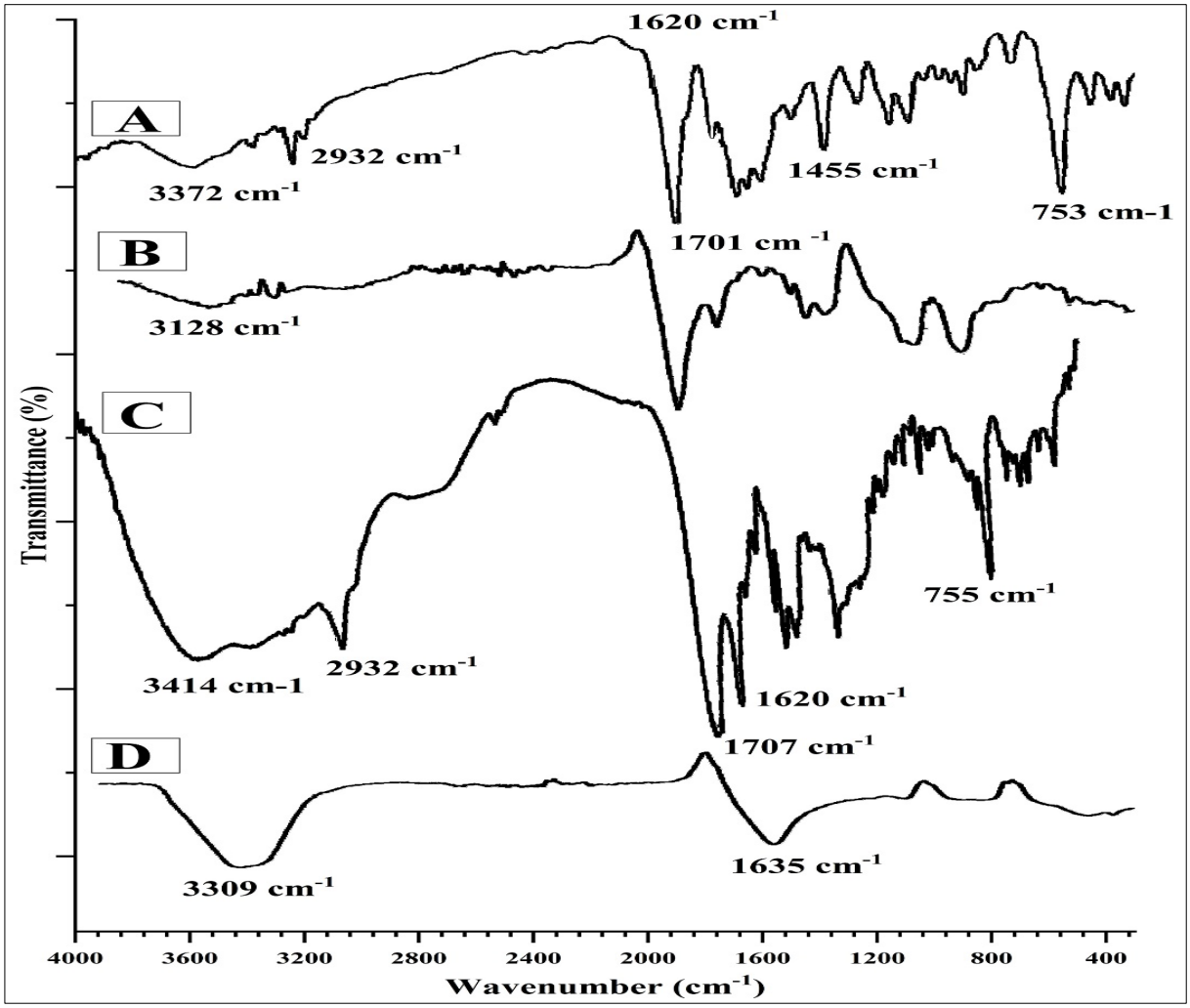

Figure 2: The FTIR; A: ONDS, B: CA (Blank), C: ONDS: CA (Physical), and D: Selected formula (F3).

2.6.2. Thermal Analysis

Differential scanning calorimeter (DSC) analysis for ONDS (free form) powder showed a sharp endothermic peak at (235 ±1 °C), as shown in (Figure 3-A). Results indicated that ONDS was a pure crystalline powder which agreed with the reported studies [28]. In addition, CA analysis did not show a sharp endothermic peak, and a valley shape at (83 °C) indicates a transition temperature of CA which was related to its amorphous properties (Figure 3-B) [29]. The physical mixture of (ONDS: CA) showed no overlapping or incompatibilities in which ONDS remained in the crystalline state (presence of sharp endothermic peak) with a slight shift to a higher temperature (242 °C). In addition, CA analysis did not show a sharp endothermic peak, and a valley shape at (83 ±0.7 °C) indicates a transition temperature of CA, which was related to its amorphous properties and also to the evaporation of water content used in the carbopol gel as shown in (Figure 3-C). The DSC thermogram of the selected formula (F3) showed a lowering of the enthalpy (ΔH) with an extensive broadening of the melting peak of ONDS, which means that ONDS crystallinity was diminished. The position of the melting peak shifted to a lower value (223.5 °C), as seen in (Figure 3-D). Reduction in melting heat (enthalpy) suggested that most ONDS entrapped within invasome vesicles in a molecularly dispersed rather than a crystalline form [30].

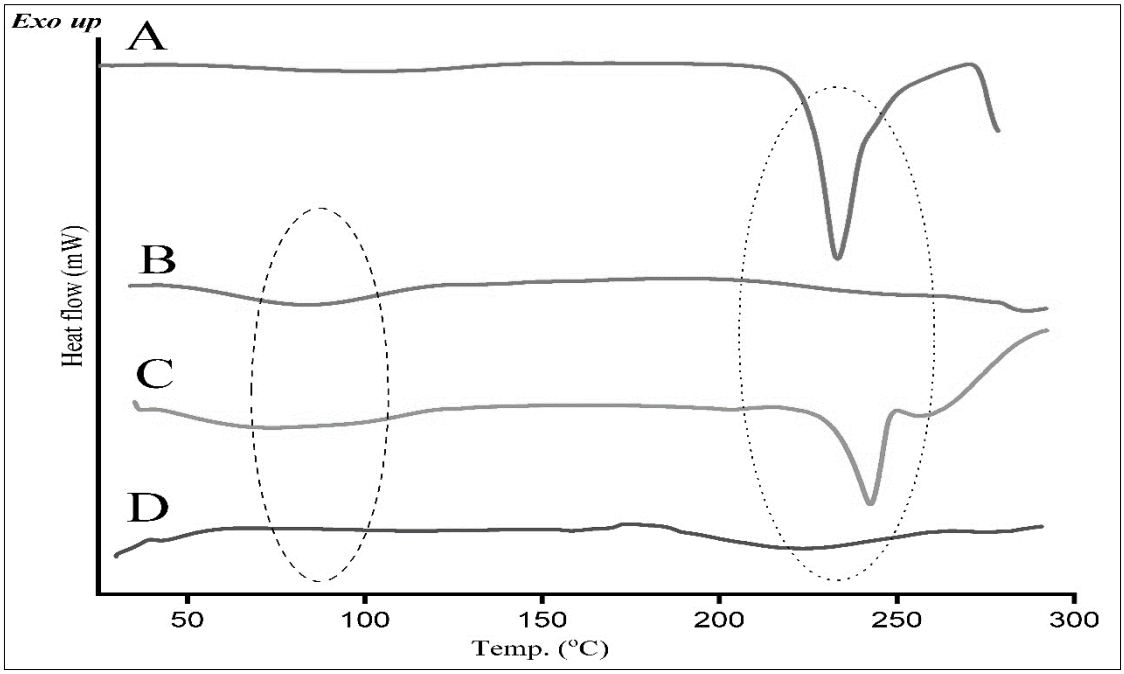

Figure 3: Differential scanning calorimetry; **A:** ONDS, **B:** Blank CA, **C:** ONDS: CA , and **D:** Selected (F3)

2.6.3. XRD

The XRD for pure ONDS is shown in (Figure 4-A), revealing sharp, high intensive peaks at diffraction angles °2 Theta (2θ) of (6°, 9.90°, 21.5°, 24°, 25.52°, 27°, 29°, and 32°), respectively which indicating that the ONDS molecule was in a pure crystalline form as shown in (Figure 4-A). In contrast, the XRD analysis of (F3) did not show any characteristic peaks of ONDS at its diffraction angles. The absence of peaks confirmed the lack of crystallinity and conversion to a dispersed state, as shown in (Figure 4-B). The loss of the crystal lattice of ONDS was driven by its entrapment in invasome vesicles, indicating its presence as a solubilized form within the vesicles' gel framework [8].

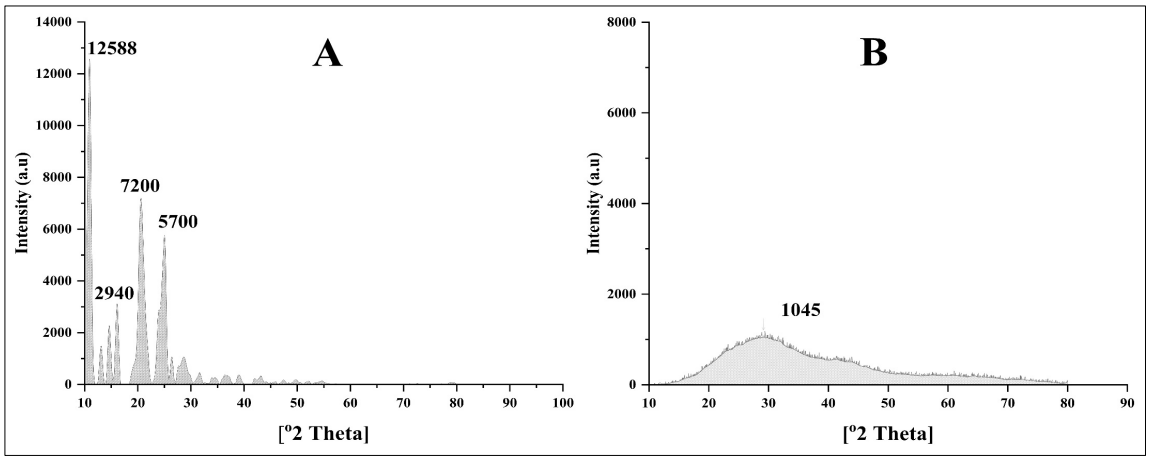

Figure 4: The x-ray diffractogram; **A:** Ondansetron and **B:** Selected (F3).

2.7. The in vivo local skin irritation and histopathology

Daily observations for six consecutive days showed that the group treated with formalin (10%, v/v) solution exhibited severe erythema (4 scores) and slight to moderate edema (2 - 3 scores). The other groups treated with blank CA gel (0.5%, w/v) (control) and with test (F3) were showed very slight erythema (0 to 1 score) and no edema, as shown in (Figure 5-I). Results revealed a significant difference in irritation ($p< 0.05$) between irritant and test treatments. In addition, no significant difference ($p> 0.05$) was observed between the control and test treatments. Generally, if a substance scores 2 or less on the irritancy scale, it is not irritating.

As a result, (F3) was considered non-irritant [7]. After six days, a histological examination was begun. Hematoxylin and eosin (H and E) stains were used to identify the prepared microscope slides. The epidermis, dermis, and subcutaneous tissues were distinct and well-defined in untreated tissue slides (arrows), as in (Figure 5, II-D) [31]. Identical observation results were found for slides obtained from the group treated with CA gel, as shown in (Figure 5, II-A). Application of the selected formula (F3) revealed no significant alterations ($p > 0.05$) in tissue structure, despite a slight enlargement in the interstitial tissues and modifications in the SC layer due to the permeation effect of invasome vesicles as shown in (Figure 5, II-B). The application of (10%, v/v) formalin aqueous solution induced symptoms of lysis of skin tissues (arrows), and SC seemed loose or peeled, as shown in (Figure 5, II-C).

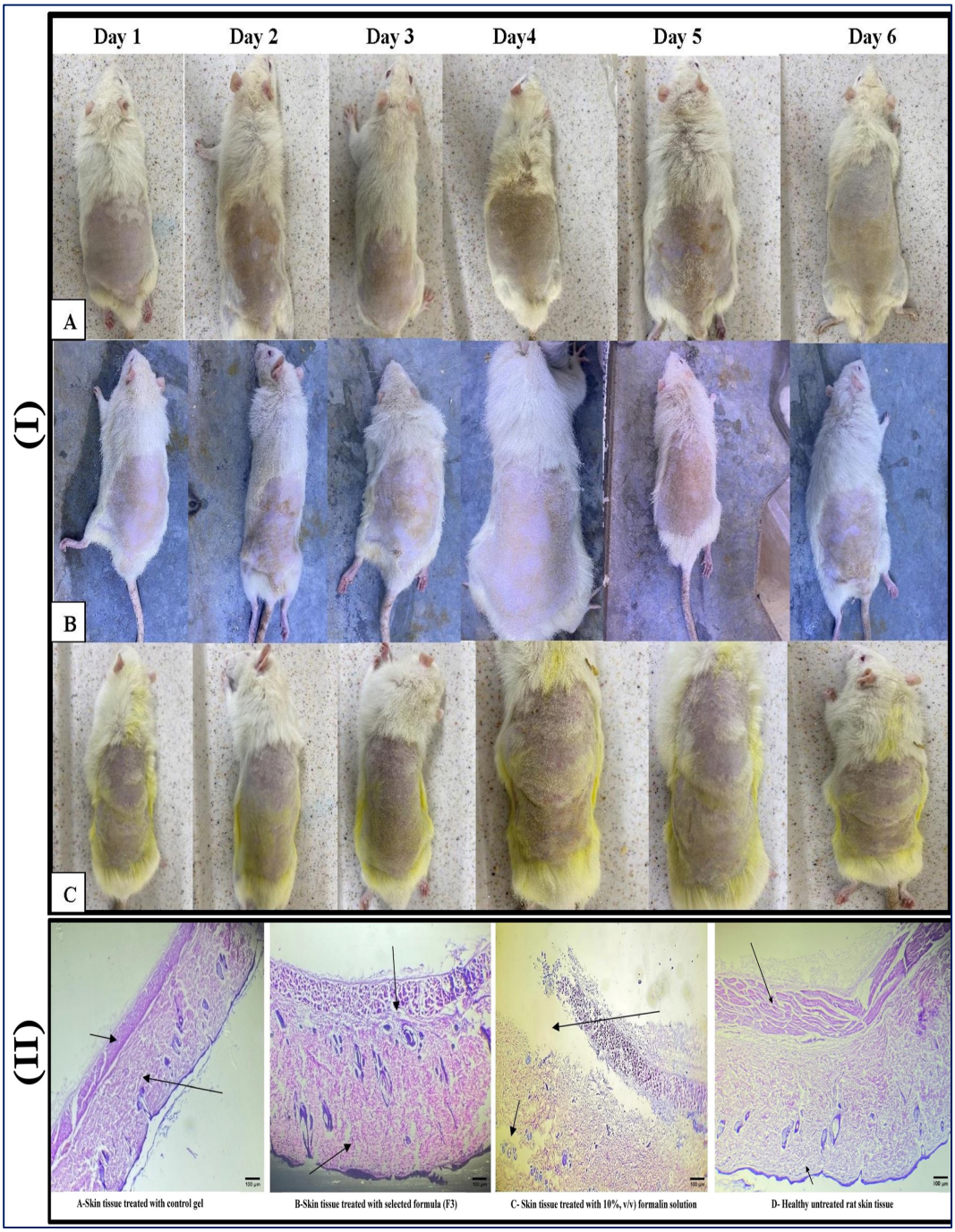

Figure 5: I) Skin irritation study; A) CA blank gel (Control), B) Test (F3) and C) (10%,v/v) formalin solution. II) Representative H and E-stained micrographs, A)Treated with control, B) Treated with F3, C) with formalin 10%,v/v) and D) Untreated healthy tissue. Staining: Hematoxylin and eosin. Bar (—): 100 µm

2.8. Characterization results

2.8.1. Field emission-scanning electron microscope (FE-SEM)

Surface morphology analysis was studied via FESEM for ONDS and CA gel and was selected (F3). As seen in (Figure 6-A), ONDS appeared as a crystalline molecule, which agrees with DSC and x-ray diffraction results. In addition, the texture of CA was homogenous and not brittle, as shown in (Figure 6-B). Invasomes vesicles dispersed within CA gel (as bright spots) had a nanosized range in size, as shown in (Figure 6-C), which agreed with vesicle size determination using Malvern® zeta sizer (Malvern, UK) that revealed mean vesicle size (Dav. = 202.9 ± 0.5 nm), polydispersity index (PDI) was (0.2 ± 0.02) as shown in (Figure 7-A), and zeta potential (ζ) was (-38.64 ± 0.13 mV) as shown in (Figure 7-B) [32]. Nanovesicles might penetrate and release ONDS more efficiently than microsized vesicles, which retain some of the vesicles in the upper layers of the skin and might cause a local release of ONDS from vesicles [33].

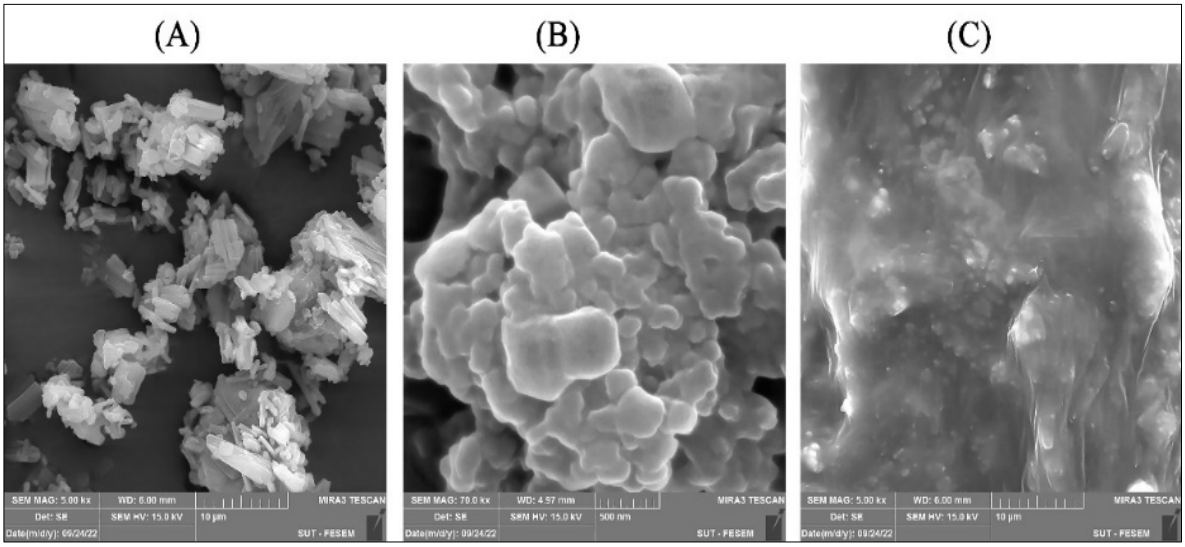

Figure 6: The FESEM; **A:** Pure ONDS**, B:** CA gel, and **C:** Selected formula (F3).

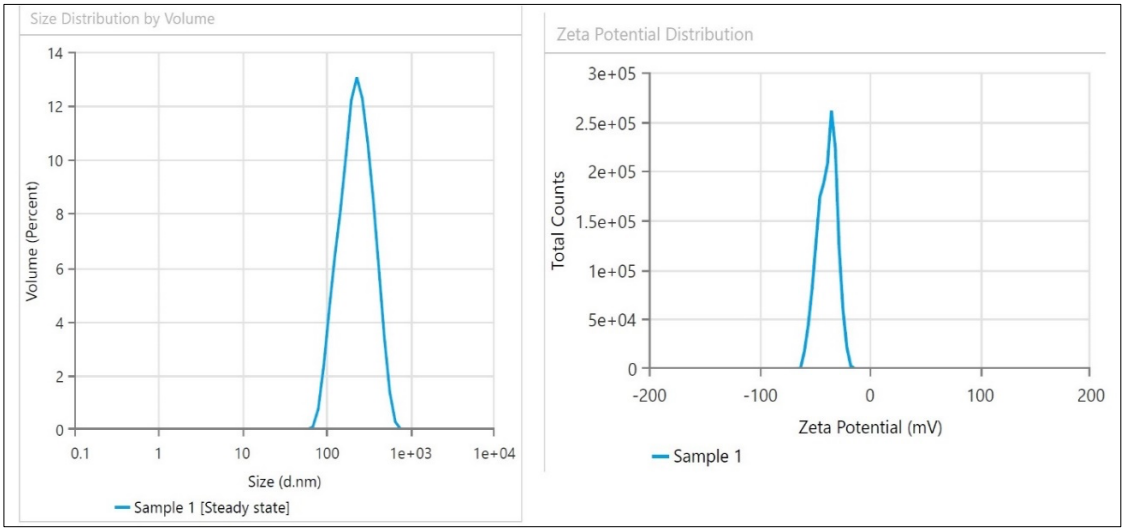

Figure 7: Zeta sizer analysis for (F3); **A:** Size distribution (nm)**, B:** Zeta potential (ζ).

2.8.2. Transmission electron microscope (TEM)

Images obtained from TEM showed that CA had a homogenous texture with no particulates or cracking, as seen in (Figure 8-A). However, selected (F3) showed a spherical, deformed shape of invasome vesicles in the nanosize range, which agreed with results obtained from zeta sizer determination (202.9 ± 0.5 nm) and FE-SEM characterization. The ONDS invasomes vesicles were dispersed in CA gel, as shown in (Figure 8-B) [34].

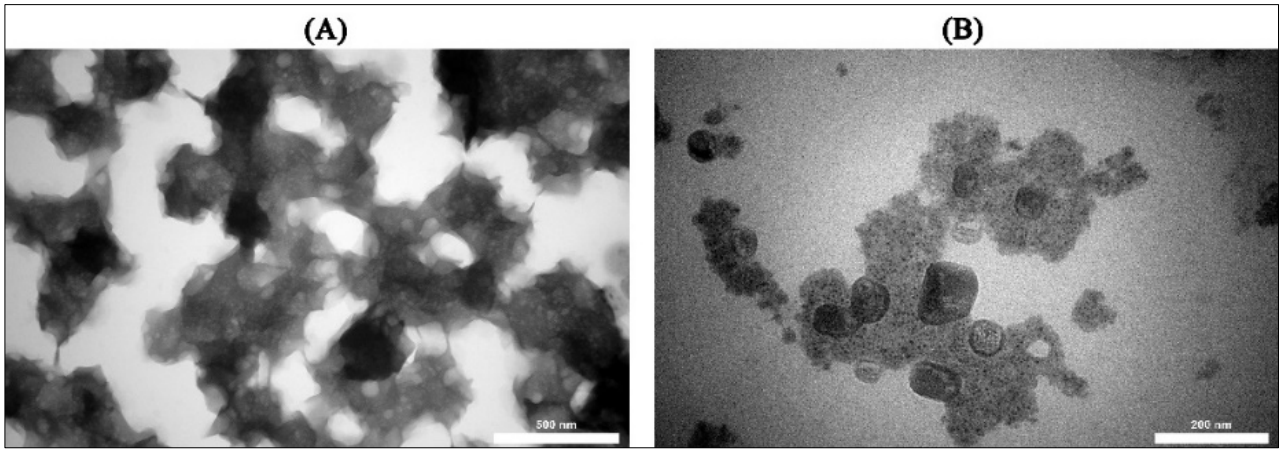

Figure 8: The TEM; **A:** CA gel and **B:** Selected formula (F3)**.**

3. CONCLUSIONS

Ondansetron-loaded invasomes gel (F3) was successfully prepared by mixing and incorporating the lyophilized ONDS invasomes powder with CA gel (0.5%, w/v) to produce (5%, w/w) gel. The selected formula (F3) was evaluated and characterized. It showed efficient ex vivo permeation and good biocompatibility with skin upon application. The study proved that ONDS entrapped in the nano-vesicles were in solubilized form, and the release of ONDS was higher than other formulas along the studied permeation time. It concluded that the developed formulation (F3) could be considered in future studies regarding stability and in vivo permeation studies using rats.

The selected formula (F3) showed (50 ± 1%) ex vivo permeation after 9 h, with no reported in vivo irritation or allergy upon application on rat skin. Therefore, Invasomes prepared as the transdermal gel is proposed as a promised dosage form for ONDS that offers extended release time than the oral route, efficient permeation across skin layers, providing a higher amount of ONDS available for systemic action with fast onset, and consequently, enhancement of ONDS therapeutic action.

4. MATERIALS and METHODS

Ondansetron (purity ≥ 99%), limonene, egg, and soybean lecithins (Hangzhou Hyper Chemicals® Ltd., Zhejiang, China). Ethanol (99%, v/v), sodium chloride, potassium chloride, potassium dihydrogen phosphate, disodium hydrogen phosphate, sodium lauryl sulfate (SLS), methanol (HPLC grade), acetonitrile (HPLC grade), carbopol 934P, HPMC K_4M and diethyl ether (Alpha Chemika®, India). Orthophosphoric acid (Isolab, Germany) and methylparaben (Fluka® Chemical AG, Switzerland). Other analytical solvents, instruments, and glassware were supplied locally.

4.1. Experimental animals

Male Wistar albino rats weighing (200 ± 20 g) were used in the study. Every 12 h, the light cycle in the animal room was inverted (lights off at 8 a.m.). The room temperature was kept at (23 ± 2 °C) with adequate ventilation and oxygen supply. The relative humidity was maintained at (50 ± 10 °C).

During the experiment, rats were kept in cages with access to a standard nutritionally balanced diet and tap water *ad libitum*. The procedures performed on the animals follow the WOAH (Formerly Office International des Epizooties) foundation for animal ethical principles in studies involving animals and animal specimens. Furthermore, the ethical committee of the College of Pharmacy/University of Baghdad (No: REAFUBCP11210204A) reviewed and approved all the procedures and protocols performed on animals by following the standards for the care and use of laboratory animals.

4.2. Preparation of ONDS invasomes gel

Invasomes loaded ONDS dispersions (1%, w/v) were prepared, optimized, and lyophilized using a freeze dryer (FreeZone, USA). The entrapment efficiency of ONDS in the selected invasomes dispersion was (88 ± 4%). Four formulas were prepared using two gelling agents (HPMC K_4M and CA at (0.5% and 1%, w/v) each. Both are pure and of pharmaceutical grade. A blank gel of HPMC K_4M was prepared by dissolving a specified weight of the polymer in a specified volume of deionized water, mixing well in a beaker using a magnetic stirrer with continuing heating at (60 ± 5 °C). The final volume was completed by deionized water

to obtain (0.5% or 1%, w/v) gel, and it was left aside for 24 h to eliminate any bubbles. On the other hand, in CA, no heating was needed for mixing. The formed solution (0.5 or 1%, w/v) was kept for 24 h in the refrigerator to allow carbopol to be thoroughly wetted by water and to get rid of bubbles. After 24 h of storage, a few drops of triethanolamine were added to the slurry with continued mixing until gel formed. Methylparaben utilized as a preservative at (0.1%, w/v) was added during the preparation of the gels. Optimized formulas shown in Table 1 were prepared by incorporating and mixing a specific weight of lyophilized ONDS-invasomes powder with a gelling agent until a homogenous, clear gel formed. The final ONDS invasomes-loaded gel concentration is (5%, w/w) [35]. Formulas were stored in a refrigerator at (8 ± 2 °C) during the study. The plain gel was prepared by dissolving (100 mg) of ONDS in (2 ml) of absolute ethanol using a bath sonicator at (25 ± 2 °C). Mixing continued until a clear solution was obtained. At a specified concentration, the gelling agent was added to the solution with stirring until a homogeneous gel formed, in which the final concentration was equal to (5%, w/w) [36].

Table 1: Prepared ONDS invasomes gel formulas (5%, w/w) [a]

Formula	Optimized ONDS invasomes dispersion composition [b]					Type of gelling agent			
	ONDS (%, w/w)	**(E: S) (%, w/v)**	**Lim. (%, v/v)**	**Abs. Eth. (%, v/v)**	**PBS (ml) Q. S**	**HPMC K_4M (0.5%, w/v)**	**HPMC K_4M (1%, w/v)**	**CA (0.5%, w/v)**	**CA (1%, w/v)**
F1	1	2:2	8	20	100	Q. S.	– [c]	–	–
F2	1	2:2	8	20	100	–	Q. S.	–	–
F3	1	2:2	8	20	100	–	–	Q. S.	–
F4	1	2:2	8	20	100	–	–	–	Q. S.

ONDS: Ondansetron, (E: S): Egg : Soybean lecithins, Lim: D-limonene, Abs. Eth: Absolute ethanol, PBS: Phosphate buffer saline, HPMC: Hydroxypropyl methylcellulose, CA: Carbopol 934P.
a (lyophilized invasomes powder equivalent to 50 mg of ONDS in 1 g gel).
b ONDS entrapment ≈ 90% (9 mg/ml of dispersion).
c not used.

4.3. Formula's appearance and pH

Blank gels and ONDS invasomes-loaded gels were examined for appearance, presence of any aggregates, and phase separation [37]. The pH measurements were performed using a pH meter. All measurements were done in triplicate.

4.4. Determination of content uniformity

Ten dosage units for each gel formula were evaluated. It involves dissolving (1 g of 5%, w/w) ONDS invasomes gel in (5 ml) ethanol. The sample was sonicated in a bath sonicator at (25 ± 2 °C) until a clear solution was obtained. Solutions were placed in test tubes, centrifuged at 4000 rpm for 5 min, filtered and suitably diluted with ethanol, and measured spectrophotometrically. Absorbance was measured using a UV-visible spectrophotometer - 1900i (Shimadzu, Japan) at 302 nm wavelength [38]. All measurements were done in triplicate.

4.5. Viscosity study

The viscosity of the prepared gels was measured at (28 ± 1 °C) using a rotational viscometer (Myr, Spain) fitted with a spindle (R6) (radius 0.75 cm). The viscometer was considered a cup and bob type. The gel was poured into a glass beaker with a (2 cm) diameter opening and (8.5 cm) height. The height of the gel in the beaker was (6 cm). Each measurement was performed in triplicate. The viscosity was measured for every rotating speed between 10 - 200 rpm [39].

4.6. Determination of spreadability

A gel weighing (2 g) was placed on a glass plate (30 × 20 cm) marked by a circle (2 cm diameter) and covered with a second glass plate. Then, a weight (100 g) was placed on the upper glass plate for (5 min), and the results of the spreading distance were measured in triplicate [40].

4.7. The ex vivo permeation study

A vertical diffusion Franz cell (15 mm diameter, 12 ml) (Copley, UK) was utilized for the study. Using a special punch, a trainer excised the skin membrane from rats with total thickness. It entails anesthetizing rats with diethyl ether inhalation, shaving their dorsal skin with an electrical clipper, and then sacrificing them.

The receptor compartment of the diffusion cell was filled with a solution of SLS (0.5%, w/v) in phosphate-buffered saline (PBS, PH 7.4) at (32 ±1 °C) to simulate the pH and temperature of the skin. A skin membrane with an effective diffusion surface area (1.76 cm2) was fixed facing upward between the donor and receptor compartments A weight of (5%, w/w) ONDS-loaded invasomes gel equivalent to (8 mg) ONDS was used in the study [41]. Sampling was done every half hour by taking (1 ml) of the receptor solution and compensating with fresh buffer to maintain the sink condition. During the test, the diffusion cell was examined to see if bubbles were present on both sides of the membrane. A modified HPLC analysis method was used to determine ONDS in samples [42]. The method was validated in terms of (linearity, specificity, precision, and accuracy) [21]. The study was achieved using UHPLC-PDA 2060C 3D apparatus (Shimadzu, Japan), and the data analysis was performed using LabSolutions® LC/GC workstation software ver.5. The mobile phase ratio (Methanol: Acetonitrile) was (50: 50) optimized in an isocratic mode for the analysis of ONDS. Samples were collected and suitably diluted in the mobile phase. The column used was Shim-pack XR-ODS II (150 mm × 3.0 mm ID, 2.2 µm particle diameter) with detection at (200-800 nm) wavelength. The cooler temperature was 5±1 °C, the oven temperature was 40±1 °C, and the injection volume was (20 µl). The process's total run time was 5 minutes, and the flow rate was optimized at (1.2 ml/min). All measurements were done in triplicate.

The target permeation flux in humans was based on the human clearance of ONDS (Cl= 21,240 ml/h) and the mean steady-state concentration of ONDS (Css= 26.2 ng/ml). The ex vivo diffusion area (1.76 cm2) was used to calculate the desired permeation flux for ONDS via equation 2 [43]. Accordingly, humans' target flux was (316 µg/cm2/h). Steady-state flux (Jss) and enhancement ratio (ER) were calculated using equations 3 and 4, respectively [34,43]:

(Eq.2) Desired flux (J) = (Css × Cl)/ (Membrane area)

(Eq.3) JSS = (Slope of the permeation curve)/ (surface area of the membrane)

(Eq.4) Enhancement ratio (ER) = (Jss test)/ (Jss plain)

Where, (Jss) test and (Jss) plain are the steady-state flux of the ONDS invasomes gel test formula and the corresponding plain gel, respectively. In addition, lag time is computed using a trendline for each formula's permeation–time curve and extrapolating the straight line to the x-axis of the curve [35].

4.8. Compatibility studies

4.8.A. FTIR analysis

Studies were done for ONDS, selected gel, ONDS: Gel physical mixture, and the selected formula using FTIR (Shimadzu, Japan). The spectrum was scanned at (4000 – 400 cm^{-1}) frequency [44].

4.8.B. Thermal analysis

Analysis was done for ONDS, selected gel, ONDS: Blank gel physical mixture, and the selected gel formula using a differential scanning calorimeter (DSC-60) (Shimadzu, Japan). Two aluminum pans were used; the first pan was for the reference (α-alumina), and the second was for the test sample. Both were crimped by compression before the test. The instrument's temperature was raised from 25 - 300 °C at a heating rate of 10 °C/min and using nitrogen gas at a 50 ml/min flow rate [45]. Thermograms were obtained and analyzed using (TA-60WS) software (Ver. 2.2) supplied by Shimadzu® Co. for data acquisition and analysis.

4.8.C. XRD analysis

The X-ray diffraction was done for pure ONDS and the selected formula using an X-ray diffractometer (Philips, USA), the operating voltage and current were (40 kV and 30 mA), respectively. Samples were scanned at 2θ from (0 - 80°) for qualitative studies, and the scanning rate was (4°/min) [5].

4.9. The in vivo local skin irritation and histopathological studies

The calculated dose of ONDS invasomes gel for rats was determined using the body weight of the rat according to the surface area ratio. A rat weighed 200 g, and a human weighed 70 kg; the surface area ratio was equal to 56 [46]. An oral dose of ONDS for humans varies from 8–32 mg/day. The total oral dose of ONDS for vomiting is approximately (16 mg). The oral dose for a rat was calculated using equation 5 [47].

(Eq.5) Oral dose (rat) × Wt. of rat (kg) = Oral dose (human)/ Surface area ratio

Oral dose (rat) = (16 mg)/(0.2 kg X 56) ≈ 1.4 mg/kg

The oral dose for a rat weighing 200 g is (0.28 mg ≈ 0.3 mg). As the oral bioavailability of ONDS is 60%, the transdermal dose is (0.18 mg ≈ 0.2 mg). Therefore, the equivalent weight of (5%, w/w) gel that contained (0.2 mg) of ONDS was (≈ 4 mg). Eighteen healthy rats weighing (200 ± 20 g) were used. They split into three treatment groups (n=6). The first group was treated with blank gel (not loaded with formulation) (Treatment A: control), whereas the second group was treated with the selected formula (Treatment B; Test). As a standard

skin irritant, (1 ml) of a formalin aqueous solution (10%, v/v) was applied to the last group (Treatment C; standard). At the time of the study, all groups were anesthetized with diethyl ether. The dorsal skin was carefully shaved, and the applied treatment was occluded tightly with adhesive plaster to avoid any loss of the applied treatment during the daytime as rats were kept in cages. After (24 h), the plasters were opened, and observations were recorded. The application was repeated for another five consecutive days. Results were interpreted according to the Draize Scoring System (DSS) illustrated in Table 2 [48].

Table 2: Draize Scoring System (DSS) to interpret in vivo skin irritation level.

Scoring	Reaction	
	Erythema	**Edema**
0	No erythema	No edema
1	Very slight erythema	Very slight edema
2	Well-defined erythema	Slight edema
3	Moderate to severe erythema	Moderate edema
4	Severe erythema	Severe edema

Rats were anesthetized and sacrificed after six days. A histological examination compared healthy, untreated rat skin tissues. The dorsal skin tissue with its total thickness was carefully removed by a trainer and kept in formalin for 24 h. After that, tissue blocks were produced and sectioned using paraffin.

They were deparaffinized at the time of examination, and slides were prepared. The slides were examined under an optical microscope at 40X magnification [7].

4.10. Characterization studies

4.10.A. Field emission-scanning electron microscope (FE-SEM)

Pure ONDS, blank gel, and the selected formula were examined for surface morphology, appearance, and size using FESEM (TESCAN a.s., HQ, Czech Republic). In brief, a drop from the sample spread smoothly over a double-sided conductive carbon tape and a platinum coating. The images of the sample were visualized at an accelerating voltage (15 kV) [49].

4.10.B. Transmission electron microscope (TEM)

The blank gel and the selected formula were evaluated for shape and size using a transmission electron microscope (TEM) (CM 120, Philips, USA) with an accelerating voltage of (100 kV). The sample was placed on a carbon-coated copper grid and left to dry for (60 s) to form a thin film at (25 ± 3 °C) room temperature. The sample was stained with 1.5% phosphotungstic acid, visualized, and photographed [34].

4.11. Statistical analysis

All experimental data were done in triplicate as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to analyze the significance level among variables, followed by the post hoc Tukey test for the mean comparison of individual groups. Analysis was performed using Origin Pro® 2023 (Origin Lab® Corporation, Northampton, MA, USA). The confidence interval was 95%, and the significance level (p) was set at ($\alpha = 0.05$). The level of statistical significance was defined as ($p < 0.05$) [50].

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