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Preparation, *In vitro,* and *Ex vivo* Evaluation of Ondansetron Loaded Invasomes for Transdermal Delivery

Omar Saeb Salih*,[1] and Entidhar J. Al-Akkam [1]

[1]Department of Pharmaceutics, College of Pharmacy, University of Baghdad, Baghdad, Iraq.

Abstract

Invasomes are considered an inventive drug delivery system for the transdermal route. It improves the permeability of drugs across the skin layers which limits the absorption of poorly permeated drugs. It is also used for enhancing the efficacy and duration of action for drugs that had first-pass metabolism in the liver requires multiple daily doses. Invasomes contain unique components (Phospholipids, terpenes and ethanol) that act as safe and effective drug permeation enhancers across skin layers. Ondansetron is a serotonin receptor antagonist used to treat vomiting associated with various clinical conditions. The current study aimed to prepare invasomes vesicles loaded with ondansetron as dispersions and optimized. Twenty-seven formulas (1% w/v) were prepared by mechanical dispersion method. They were optimized by studying the effect of different variables on the entrapment of ondansetron in the invasomes vesicles. The optimized formulas showed higher entrapment efficiency and they were selected for further studies. Vesicle size measurements, polydispersity index, zeta potential (ζ), *in vitro* ondansetron release from vesicles and *ex vivo* permeation study using a skin of male rats, were performed. The selected dispersion was (F25), which showed (88.24 ±4.04%) entrapment efficiency, (317.7±٤.1 nm) vesicle size, (0.29±0.05) polydispersity index and (-31.5±1.6 mV) zeta potential (ζ). The *in vitro* release of ondansetron from invasomes vesicles showed that the dispersion (F25) releases (75.32±2.5%) of ondansetron after (12) h. Release data showed the Korsmeyer-Peppas kinetic model (R^2=0.99) associated with anomalous diffusion (n=0.8±0.02). An *ex vivo* permeation study revealed the steady-state flux (J_{ss}) was (340.3±14.7 µg/cm^2.h) with (0.46±0.1 h.) lag time. Invasomes could be considered a promising delivery system for enhancing the transdermal permeation of ondansetron.

Keywords: Invasomes; Lecithin; Ondansetron; Terpenes; Transdermal.

تحضير في المختبر وتقييم خارج الجسم الحي للانفاسومات المحملة بالاوندانسيترون للتوصيل عبرالجلد

عمر صائب صالح *،[١] و انتظار جاسم محمد[١]

[١]فرع الصيدلانيات، كلية الصيدلة، جامعة بغداد، بغداد، العراق.

[٢]فرع الصيدلانيات، كلية الصيدلة، جامعة بغداد، بغداد، العراق.

الخلاصة

تعتبر الأنفاسومات نظام مبتكر لتوصيل الأدوية عبر الجلد. تعمل على تحسين نفاذية الأدوية عبر طبقات الجلد الذي يحدد من أمتصاص الأدوية التي تمتاز بنفاذية ضعيفة. تستخدم أيضا لتعزيز الفعالية ومدة العمل للأدوية التي تعاني من الأيض المبكر في الكبد والذي يتطلب جرعات يومية متعددة. الإنفاسومات تحتوي على مكونات فريدة (الفوسفوليبيدات والتيربينات والإيثانول) التي تعمل كمحسنات آمنة وفعالة لنفاذية الأدوية عبر طبقات الجلد. الأوندانسيترون هو مضاد لمستقبلات السيروتونين يستخدم لعلاج القيء المرتبط بحالات سريرية متنوعة. تهدف الدراسة الحالية إلى تحضير حويصلات الانفاسومات محملة بدواء الأوندانسيترون على شكل تشتات وتحسينها. تم تحضير(٢٧) صيغة (١٪ وزن/حجم) بطريقة التشتيت الميكانيكي. تم تحسين الصيغ من خلال دراسة تأثير متغيرات مختلفة على انحباس الأوندانسيترون في حويصلات الإنفاسومات. الصيغ المحسنة أظهرت كفاءة انحباس عالية وقد تم اختيارها لإجراء دراسات أضافية. قياس حجم الحويصلات ، مؤشر التشتت المتعدد ، جهد الزيتا (ζ) ، تحرر الاوندانسيترون مختبريا من الحويصلات ودراسة النفاذية خارج الجسم الحي بأستعمال جلد الفئران أنجزت للصيغ المحسنة. تم أختيار التشتت (ف٢٥) الذي أظهر (٨٨,٢٤ ±٤,٠٤٪) كفاءة أنحباس، (٣١٧,٧ ±٤,١ نانومتر) حجم الحويصلة، (٠,٢٩±٠,٠٥) مؤشر التشتت المتعدد ، (-٣١,٥±١,٦ ميللي فولت) جهد الزيتا. التحرر المختبري للأوندانسيترون من حويصلات الأنفاسومات أظهر أن التشتت (ف٢٥) حرر (٧٥ ±٢,٥%) بعد (١٢) ساعة. بيانات التحرر أظهرت أن نموذج الحركية هو كورسماير-بيباس (ر٢=٠,٩٩) المرتبط مع أنتشار غير قياسي (ن= ٠,٨±٠,٠٢). دراسة النفاذية خارج الجسم الحي كشفت أن التدفق كان (٣,٣٤٠ ± ٧,١٤مايكروغرام\ سم٢. الساعة) مع زمن تأخير(٠,٤٦±٠,١ ساعة). الإنفاسومات يمكن اعتبارها نظام توصيل واعد لتعزيز نفاذية الاوندانسيترون عبر الجلد.

الكلمات المفتاحية: الأنفاسومات ، ليسيثين ، اوندانسيترون ، التربينات ، عبر الجلد

Introduction

The transdermal route is an essential route that overcomes many challenges in drug delivery because the drug must permeate the outer hard stratum corneum layer of the skin which is considered a limiting step in the permeation of drugs [(1)]. Drugs used for transdermal delivery should have several requirements to be a candidate for this route. Drugs should be lipophilic (log $K_{o/w}$=1-4), have a low melting point (<200ºC, low molecular weight (<500 Daltons), short half-life (<10 h.), and low dose (<20 mg/day) [(2)].

*Corresponding author E-mail: omar.abbas@copharm.uobaghdad.edu.iq
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Invasomes are newly developed types of vesicles discovered by Dr. Alfred Fahr in 2016. These vesicles are considered modified liposomes, ethosomes, and transfersomes in structure composition. Invasomes consist of phospholipids (e.g., egg lecithin, soybean lecithin) as the building block for the structure of the vesicles and a low percentage of ethanol when compared to ethosomes which avoid or minimize the local irritation effect caused by a large percentage of ethanol.

Invasomes are unique from other vesicles in the presence of terpenes. These are natural components in plants and are present either as monoterpenes like cineol, fenchone, D-Limonene, and α-Pinene or as diterpenes.

These terpenes consist of hydrocarbons and have different lipophilic-hydrophilic properties. Previous researches showed that terpenes facilitate the transdermal permeation of pharmaceuticals in dermal formulations and have a synergistic effect with ethanol and phospholipids [(3)].

Ondansetron (ONDS) is a serotonin receptor (5-HT_3) antagonist used for the treatment of vomiting during chemotherapy, post-operation, and pregnancy-associated emesis (Hyperemesis gravidarum) [(4)].

Ondansetron is a weak base (indole and piperazine rings), an amphoteric molecule with pk_a 7.4, making it soluble in acidic solutions through the formation of salt. According to BCS, it is considered a class II drug (Low soluble, high permeable). It has a molecular weight of 293 g/mol and a logP value of 2.07. Oral bioavailability is only 60% due to the extensive first-pass effect in the liver via hepatic enzymes, the melting point is 230-233 ^oC and the half-life is 3-4 h. Oral ONDS-marketed formulations cause a bitter taste in the mouth and most patients especially children refuse to take the medication. In cases such as chronic emesis, the patient is unable to take the medication via the oral route [(5)].

Patients receiving chemotherapy had multiple emesis episodes and until now those patients were treated via multiple daily I.V and I.M injections of ONDS which causes pain through these invasive routes and most of the patients are unable to tolerate I.M injection route because of low body mass, low immunity due to cancer and increase the incidence of injection site damage that may lead to secondary infection like sepsis and death.

Ondansetron is considered a good candidate to be formulated for the transdermal route. Previous studies using different approaches were performed for transdermal delivery of ondansetron to enhance permeation and improve therapeutic efficiency.

Ondansetron was formulated as polymeric nanoparticles loaded in microneedles and results showed the release profile of ONDS from nanoparticles was dependent on the type and concentration of the polymers used. The *ex vivo* skin permeation study using rat's skin showed that nanoparticles permeate more efficiently than an aqueous solution of ONDS across the skin by approximately two folds [(6)].

Studies showed that ondansetron was formulated as bilosomes vesicles and delivered transdermally for effective permeation and systemic effect. Results showed bilosomes system had a spherical non-aggregating appearance, good ONDS entrapment efficiency, and improved ONDS permeation compared to the ONDS solution [(7)]. Another study reported using ethosomes and transfersomes as vesicles for the delivery of ONDS transdermally.

Results showed that the prepared ONDS transfersomes were proved to be better than ethosomes. It also showed good deformability and optimized results in transdermal permeation through rat skin [(8)].

The current study aims to prepare ONDS loaded invasomes vesicles as dispersions which are considered the first step in the formulation of the transdermal dosage form. Selected optimized dispersions were based primarily on higher entrapment efficiency of ONDS in the vesicles and then, other evaluations were performed for the optimized dispersions. They evaluated for their vesicles size, polydispersity index, zeta potential (ζ), *In vitro* dissolution, and *ex vivo* permeation study. The dispersions that showed higher entrapment of ONDS, acceptable vesicle size in the nano-size range with higher amount released, and higher permeation flux across rat skin dorsal membrane were considered for further study, characterization, and optimization.

Materials and Methods

Ondansetron, lecithin (egg lecithin and soybean lecithin), terpenes (β-Citronellol, α-Pinene, and D-Limonene) all purchased from Hangzhou® Hyper Chemicals Limited, Zhejiang, China. Absolute ethanol (99% v/v), sodium chloride, potassium chloride, potassium dihydrogen phosphate, disodium hydrogen phosphate, sodium lauryl sulfate (SLS), methanol, and acetonitrile (HPLC) grade purchased from Alpha® chemika, India, and dialysis membrane 70 nm pore size purchased from Himedia®, India. Other analytical solvents, instruments, and glass wares were supplied by the College of Pharmacy / University of Baghdad for the accomplishment of this research study.

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*. Furthermore, the animal study complied with the ethics as reported in the

guidelines written by the National Committee for Research Ethics in Science and Technology (NENT), Norway [9].

Preparation of ondansetron loaded invasomes dispersions

Ondansetron invasomes dispersions were prepared by a mechanical dispersion method [10]. Twenty-seven formulas (F1 to F27) were prepared with different compositions as illustrated in Table. 1. A vortex mixer (Heidolph, Germany) was used for mixing ONDS, lecithin, terpene, and ethanol until a clear solution formed. The solution was kept in a bath sonicator (Power Sonic 410, Korea) at 70 ±5˚C for 15±2 minutes, then vortexed along with the addition of phosphate-buffered saline (PBS) (pH 7.4) at 70 ±5˚C to the solution drop by drop from a needle syringe at a constant rate until the total volume of the dispersion reached 5 ml. The prepared invasomes dispersions were sonicated via probe sonicator (QSONICA, USA) for 8 min. with an interval of 2 sec. pulse cycle and amplitude of 20±2%. The prepared invasomes dispersions were stored in the refrigerator at 4±1ºC and used the next day for evaluation studies.

Table 1. Prepared ONDS loaded invasomes Dispersions (10 mg/ml) [a]

Formula ingredients	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**
ONDS (% w/v) [b]	1	1	1	1	1	1	1	1	1
(E:S) (% w/v) [c]	2:2	4:4	8:8	2:2	4:4	8:8	2:2	4:4	8:8
Terpene (% v/v)	2	2	2	4	4	4	8	8	8
Terpene type [d]	β- Citr.	β- Citr.	β- Citr.	β- Citr.	β- Citr.	β- Citr.	β- Citr.	β-Citr	β- Citr.
Abs. Eth. (% v/v) [e]	20	20	20	20	20	20	20	20	20
Formula ingredients	**F10**	**F11**	**F12**	**F13**	**F14**	**F15**	**F16**	**F17**	**F18**
ONDS (% w/v)	1	1	1	1	1	1	1	1	1
(E:S) (1:1) (% w/v)	2:2	4:4	8:8	2:2	4:4	8:8	2:2	4:4	8:8
Terpene (% v/v)	2	2	2	4	4	4	8	8	8
Terpene type	α-Pin.	α-Pin.	α-Pin.	α-Pin.	α-Pin.	α-Pin.	α-Pin.	α-Pin.	α-Pin.
Abs. Eth. (% v/v)	20	20	20	20	20	20	20	20	20
Formula ingredients	**F19**	**F20**	**F21**	**F22**	**F23**	**F24**	**F25**	**F26**	**F27**
ONDS (% w/v)	1	1	1	1	1	1	1	1	1
(E:S) (1:1) (% w/v)	2:2	4:4	8:8	2:2	4:4	8:8	2:2	4:4	8:8
Terpene (% v/v)	2	2	2	4	4	4	8	8	8
Terpene type	D-Lim.	D-Lim.	D-Lim.	D-Lim.	D-Lim.	D-Lim.	D-Lim.	D-Lim.	D-Lim.
Abs. Eth. (% v/v)	20	20	20	20	20	20	20	20	20

a Phosphate buffer saline (PBS) (pH 7.4) q.s. ad. to 5 ml (100% v/v), **b** ONDS: Ondansetron, **c** (E:S): Egg: Soybean lecithins (1:1) amount ratio, **d** β- Citr.: β- Citronellol, α-Pin.: α-Pinene, D-Lim.: D-Limonene, **e** Abs. Eth.: Absolute ethanol (99% v/v).

Determination of ondansetron-saturated solubility in different media

Ondansetron solubility in absolute ethanol, phosphate-buffered saline (PBS) (pH 7.4), terpenes (β-Citronellol,α-Pinene, and D-Limonene), and solution of SLS in PBS (0.5% w/v) was determined by shake flask method using water bath shaker at 37 ±2ºC (adjusted by thermometer periodically). An excess amount of ONDS was added to 10 ml media in a test tube and kept in the shaker for 72± 0.5 h. After, test tubes of different media were taken and centrifuged at 6000 rpm for 15 min. and the supernatant of the separated clear solution was withdrawn via a syringe, filtered through a microfilter (0.22µm) to get rid of any undissolved particles of ONDS and suitably diluted by one of the tested solvents prior measurement [11]. In the case of terpenes, the supernatant was diluted with absolute ethanol. As they are oils, ethanol is chosen as the optimum solvent for these terpenes. The obtained solution was filtered, and suitably diluted with absolute ethanol prior to measurements [12]. After serial dilutions, the absorbance of ONDS was measured using a UV spectrophotometer (Shimadzu, Japan) at a specified wavelength for each media. All measurements were done by recording the absorbance in each medium. The concentration of ONDS in each medium was calculated from the constructed calibration curve of ONDS in each one (from the equation of the regression line). All the measurements were done in triplicate to obtain the mean of three readings± Standard deviation (SD).

Determination of ondansetron entrapment efficiency

Ondansetron entrapment efficiency (EE%) was measured by direct method using (1 ml) of the prepared dispersions which contained theoretically (10 mg) of ONDS. It involves separation of the aqueous phase (PBS layer) from the phospholipid layer using refrigerated centrifugation (Eppendorf, Germany) at 4 ±0.5 ºC with 18000 ±1000 rpm rotation speed for 30 ±5 min. repeated twice and then at 16000 ±1000 rpm rotation speed for 15± 2 min once. The separated lipid layer was dissolved in (25 ml) absolute ethanol. Samples from this volume

were drawn by syringe, filtered by microfilter (0.22µm pore size) and suitably diluted for measurement UV absorbance at 302 nm [13]. The amount of ONDS in the lipid layer was calculated from the calibration curve previously constructed using absolute ethanol and the entrapment efficiency was calculated using Equation 1. All measurements of UV absorbance of ONDS were done in triplicate.

$$Entrapment\ efficiency\ (EE\%) = \frac{Amount\ of\ ONDS\ in\ the\ lipid\ layer}{Theortical\ amount\ of\ ONDS\ used}\ x\ 100\% \quad \text{(Equation 1)}$$

Study vesicle size, polydispersity index (PDI) and zeta potential (ζ)

Optimized invasomes dispersions with higher entrapment efficiency (≥80%) were evaluated for vesicle size, polydispersity index (PDI), and zeta potential (ζ) measurements using Malvern® Zeta Sizer (Malvern, UK) [14]. Samples were properly diluted with distilled water and measured in the instrument using a quartz cuvette via dynamic light scattering at 25ºC with a scattered back angle 175º. All measurements were done in triplicate [15].

Study the release of ONDS from the optimized dispersions via in vitro dissolution

Optimized dispersions were evaluated for the *in vitro* dissolution via dissolution apparatus paddle type II (Faithful, China) using a dialysis membrane 70 nm pore size. Dialysis bags were papered by enclosing one end of the membrane with wire rubber, filled by a syringe with an adequate volume of the prepared optimized dispersion then enclosed from the other end by wire rubber to form bags that resemble that of chocolate candy as shown in Figure. 1, in which the volume of the optimized dispersion used in each bag was equivalent to (10 mg) of ONDS [6]. The dialysis bags were placed inside the dissolution jars that contained 500±5 ml PBS (pH 7.4) at 37±1 ºC and paddles were rotated at 50 rpm speed. Samples were taken from the jars, replaced by fresh buffer at specified intervals, filtered by microfilter (0.22µm pore size), suitably diluted with PBS and measured spectrophotometrically at 310 nm. All measurements were done in triplicate [5,6]. Studying the *in vitro* dissolution for the optimized dispersions was done, analyzed, and compared for any similarity among dispersions. Dissolution kinetic modeling for the selected dispersion was done using the DDSolver® add-ins program in Microsoft® Excel 2019 [16].

Figure 1. Dialysis bag at the bottom of the dissolution jar using weight for settling

Study the permeation of ONDS from optimized dispersions via ex vivo diffusion (Transdermal flux)

A vertical diffusion cell, HDT 1000 (Copley, UK) applied for the *ex vivo* diffusion study in which the receptor compartment used was 12 ml volume and 15 mm opening diameter with a screw-type sample holder. The study was done at 32 ±1 ºC, in which the cell was filled carefully in the receptor compartment with a solution of SLS in PBS (0.5% w/v) to achieve sink condition and use a magnetic stirrer bar inside the cell for rotating the solution during the study in which it set to rotate at 600 rpm [17]. Skin membrane from Wistar male albino rats obtained by aid from a trainer. It involves anesthetizing the rats with inhalation by diethyl ether fumes, removing hair from dorsal skin, and sacrificing by excising the dorsal skin membrane without damaging the stratum corneum using a specialized punch accessory that had a 1.76 cm^2 surface area. Skin membranes were taken and carefully fixed between the donor and receptor compartments in which the stratum corneum faced upward and the donor compartment was an open system type. The effective surface area of the used cell for diffusion was (1.76 cm^2) so the skin membrane was cut optimally to suit that area [8]. Optimized dispersions were evaluated for a permeation study in which a volume equivalent to (10 mg) of ONDS was taken and added to the donor compartment. All measurements were done in triplicate.

Samples were withdrawn from the cell via syringe, replaced by a fresh receptor medium periodically, filtered via microfilter (0.22µm pore size) and were analyzed by UHPLC-PDA 2060C 3D (Shimadzu®, Japan) by a method reported by Dutta with some modifications in the method of analysis [18].

The method was validated for linearity by constructing a calibration curve, accuracy, precision, and limit of detection before using it for the determination of ONDS in an *ex vivo* permeation

study. Data analysis was performed using LabSolutions® workstation software ver. 5 which was supported by Shimadzu®. The mobile phase ratio (Methanol: Acetonitrile) was optimized in an isocratic mode for the analysis of ONDS to (50:50). The column used was Shim-pack XR-ODS II (150 mm × 3.0 mm I.D., 2.2 µm particle diameter) with detection at (200-800 nm) wavelength. The cooler temperature was set at 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±0.1 min. and the flow rate optimized at (1.2 ml/min). After the complete run for 5 min., the retention time was 3 min as shown in Figure. 2. All measurements were done in triplicate.

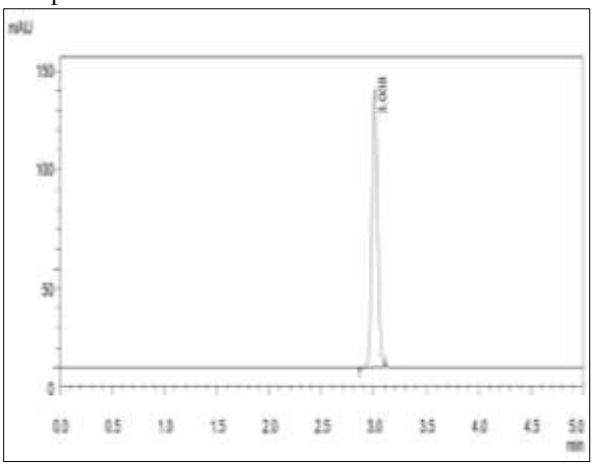


Figure 2. Chromatogram of ONDS at pH (3± 0.02) by UHPLC PDA-UV detector, n=3

The calculations for obtaining the target flux (rate of permeation) were based on the pharmacokinetics parameters of ONDS. It depends on ONDS clearance (Cl= 21.24 L/h.) and the mean steady-state plasma concentration of ONDS (C_{ss}= 26.2 ng/ml). The target flux for ONDS using (1.76 cm^2) membrane (from the Franz cell) was 316 µg/cm^2.h. which is calculated by equation 2 [19]. The studied transdermal flux for ONDS-loaded invasomes dispersions should be equal to this value when considering it in further evaluations and future formulations.

$$\textit{Target flux } (J_{ss}) = \frac{Css\ X\ CL}{Area\ of\ membrane} \quad \textbf{(Equation 2)}$$

Due to their abundance, portability and low cost, the skin of rodents is commonly used for *ex vivo* percutaneous permeation experiments. Rat skin is the most popular rodent model because of its structural similarities to human skin [20]. The slope obtained from the graph between the amount of ONDS permeated per surface area (cm^2) and time (h.) was used to calculate the desired flux (J_{ss}) using equation 3 and the permeability coefficient was calculated by using equation 4 [21]:

$$\textit{Desired flux } (J_{ss}) = \frac{Slope\ of\ the\ curve}{surface\ area\ of\ the\ membrane} \quad \textbf{(Equation 3)}$$

$$\textit{Permeability coefficient } (K_p) = \frac{Jss}{Cd} \quad \textbf{(Equation 4)}$$

In which C_d is ONDS's initial concentration at the donor compartment. The area of application on human skin needed to achieve a steady state concentration (C_{ss}) of ONDS was calculated by equation 5 using the obtained (J_{ss}) data of the selected dispersion. The total daily transdermal dose of ONDS is (14.5 mg) which is obtained from the total daily oral dose (24 mg) used by adults for the treatment of vomiting multiplied by the % oral bioavailability of ONDS (60%) [19].

$$\textit{Area of application} = \frac{Daily\ transdermal\ dose \times 3}{t \times Jss} \quad \textbf{(Equation 5)}$$

Where (t) is the time for which the steady-state concentration should be maintained (for a single daily transdermal dose, t = 24 h.), (J_{ss}) is the observed flux using excised rat skin tissue and (3) is the correction factor to calculate the surface area needed for adequate permeation of ONDS in human based on the (J_{ss}) obtained from the excised dorsal rat skin [19]. Enhancement ratio (ER) also could be calculated by using equation 6 which demonstrated the number of times enhancement in permeation when compared to a control formulation [22,23].

$$\textit{Enhancement ratio } (ER) = \frac{Jss\ of\ test}{Jss\ of\ control} \quad \textbf{(Equation 6)}$$

In which (J_{ss}) of the test is the steady state flux of the optimized ONDS loaded invasomes dispersion and (J_{ss}) of control is the steady state flux of plain dispersion of (10 mg/ml) ONDS in PBS (pH 7.4). Additionally, lag time (T_{lag}) is the time taken for permeation to reach a steady state. It is calculated from the X-axis intersected with the extrapolated line from the linear portion of the permeation curve. The optimized dispersions were evaluated in accordance with their (T_{Lag}).

Visualization of invasome vesicles by transmission electron microscope (TEM)

The selected best ONDS loaded invasomes dispersion was visualized for shape and vesicle size using TEM (CM 120, Philips, USA) with an accelerating voltage of 100 kV which was done by placing a sample on a carbon-coated copper grid. The sample was stained with 1.5% phosphotungstic acid for identification purposes and then visualized [24].

Statistical analysis

Results obtained from the experimental work were demonstrated as mean ± standard deviation (SD) of three measurements. One-way analysis of

variance (ANOVA) was employed to examine the significance among different formulas. The level of statistical significance was defined as (p<0.05). DDSolver® add-ins program was used for dissolution data modeling analysis [25].

Results and Discussion

Ondansetron saturated solubility

The solubility of ONDS in different media is shown in Figure. 3. the structure of the ONDS molecule, contains chemical groups that affect the solubility properties of ONDS. It is considered an amphiphilic molecule (logP =2±0.1). Solubility results showed that it was soluble in absolute ethanol due to the presence of the non-polar group in the molecule that solvated with the non-polar group of ethanol. The study of the solubility of ONDS in absolute ethanol was carried out to identify the solubilization capacity of ethanol and to decide whether to use or not as a solvent during the study. Results showed that it is considered an efficient solvent and it could be used as a solvent for ONDS amount determination in entrapment efficiency study without the need for other solvents.

The study of solubility in PBS was crucial to identify the solubility of ONDS in the study of *in vitro* dissolution in which sink condition should be achieved during the experiment and ensure ONDS solubilized in the dissolution medium. Results showed that ONDS was very slightly soluble in PBS at pH 7.4 [26], which agreed with previous research that studied the effect of raising pH on ONDS solubility [5]. Due to the basic character of the ONDS molecule that is related to the presence of indole and piperazine rings, it will be in the ionized form in the acidic medium (pH less than 7), ONDS solubility at acidic pH was significantly (p<0.05) higher than at alkaline pH (pH more than 7). As pH rises, the unionized species outnumber the ionized species, decreasing the apparent solubility at higher pH [27]. The volume of BPS used in the *in vitro* dissolution study was adequate to maintain sink condition as the volume used was 500±5 ml in a vessel of 900 ml capacity. In contrast, the buffer used in the *ex vivo* permeation study was very low (12±0.2 ml) that change the solubility of ONDS when used in the same amount as that in the dissolution study. To avoid non sink condition, either use small amount of ONDS in the study (which was not applicable) or the use of buffer mixed with adequate ratio of surfactant that used to enhance the solubilization of ONDS and avoid saturation. Sodium lauryl sulfate (SLS) at (0.5% w/v) was an optimum surfactant chosen among others and used for this purpose. The study of solubility in (0.5% w/v) SLS/PBS solution (pH 7.4) was performed.

The addition of SLS increased the apparent solubility of ONDS significantly (p<0.05) by ten times from that when using buffer alone at the same pH. It was employed as a surfactant to increase the dissolution rate of ONDS particles in the buffer [21].

Solubility of ONDS in terpenes was also carried out to identify the suitable terpene that ensures optimum solubilization of ONDS during the formulation process. Results showed that ONDS solubility in terpenes was 28 ±2.02 mg/ml in β-Citronellol, 37 ±5.05 mg/ml in α-Pinene and 49 ±3.08 mg/ml in D-Limonene, respectively. Statistical analysis using one-way ANOVA showed a significant difference (p<0.05) among the terpenes used on the solubility of ONDS. More ONDS solubilized when D-Limonene was used. The solubilization efficiency of terpenes was related to their lipophilicity properties which in turn related to their partition coefficient value. The higher, the partition coefficient, the higher the lipophilicity of the terpene. Data showed from literature the partition coefficient of the used terpenes was (LogP=3.2 for β-Citronellol, LogP=2.8 for α-Pinene and logP=4.83 for D-Limonene) [28].

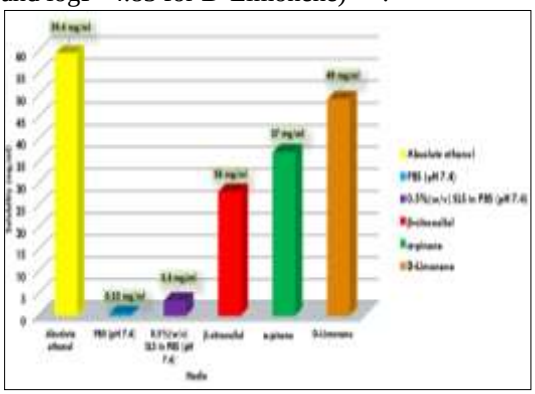


Figure 3. Solubility of ONDS in different media, n=3

Effect of invasome components on ondansetron entrapment efficiency

1. Effect of type and percent of terpene used

As noticed in Table. 1, the terpenes used in the prepared ONDS loaded invasomes dispersions from (F1-F9) were β-Citronellol, from (F10-F18) used α-Pinene and (F19-F27) used D-Limonene. Results of entrapment efficiency shown in Table. 2 were ranged from (62.1 ±3.05%-82.65 ±2.07%) using β-Citronellol, (63.18 ±1.02%-83.45 ±2.04%) using α-Pinene and (68.1 ±1.08%-88.24 ±4.04%) using D-Limonene, for the prepared dispersions respectively. Results showed a significant difference (p<0.05) in the percent of entrapment of ONDS upon the effect of the type of terpene used. Data showed that D-Limonene had higher entrapment efficiency, as shown in (F22 and F25), which might be related to the higher lipophilicity of D-Limonene that were expected to solubilize ONDS more than other terpenes [29].

On the other hand, different percentages of terpene were used in the preparation of ONDS loaded invasomes dispersions (2%, 4%, and 8% v/v) as shown in Table. 1 with a constant % of other ingredients in the vesicles. Results showed that the

EE% was increased with an increase in % of terpene used regardless of its type.

As seen in Table. 2 (F1, F4, and F7) which contained β-Citronellol they showed EE% (72.56 ±1.07%, 80.5 ±5.02% and 82.65 ±2.07%), respectively. For (F10, F13 and F16) which contained α-Pinene, they showed EE% was (75.5 ±3.03%, 80.5 ±8.08% and 83.45 ±2.04%), respectively. For (F19, F22 and F25) which contained D-Limonene they showed EE% (79.45±5.06%,85.23±5.03% and 88.24±4.04%), respectively.

Results revealed no statistically significant difference (p>0.05) for dispersions containing β-Citronellol as in (F1, F4, and F7) and α-Pinene in (F10, F13, and F16), while results showed a significant effect (p<0.05) for dispersions contained D-Limonene (F19, F22, and F25). The reason was the lipophilic properties of D-limonene used and its association with the phospholipids in the invasomes vesicles which agreed with the obtained results and were considered one of the factors that improve entrapment efficiency [30].

2. Effect of percent of lecithins mixture used

The effect of increasing % of lecithins mixture (Egg: Soybean) amount ratio used in the preparation of dispersions on EE% showed in Table. 2 in which lecithins were used in 3 levels (2:2, 4:4 and 8:8) % w/v.

Results showed for dispersions (F1, F2, and F3), the EE% was 72.56 ±1.07%, 65.44 ±2.05%, and 62.1 ±3.05%, respectively. For dispersions (F10, F11 and F12), the EE% was 75.5 ±3.03%,68.34 ±4.08% and 63.18 ±1.02%, respectively and for dispersions (F19, F20 and F21), the EE% was 79.45 ±5.06%,73.22 ±2.07% and 68.85 ±1.02%, respectively. Results indicated that the EE% of ONDS significantly decreased (p<0.05) with an increase in the % of lecithins used in the presence of a constant % of other ingredients. One of the explanations for this effect was the amphiphilic nature of the ONDS molecule as it incorporated into the invasomes vesicles between the lipophilic chains of the phospholipid molecules. At a low percent of lecithins, ONDS could be completely incorporated into the bilayer membrane, which explains the high encapsulation efficiency at lecithin ratio (2:2) %w/v while at a higher percent (8:8) %w/v, the lipophilic chains of phospholipids were overlapped which made ONDS difficult to be incorporated and a portion of ONDS particles may be settled on the surface of vesicles rather than entrapped inside the vesicles which could be lost during the formulation process [31].

Table 2. Entrapment Efficiency (EE%) Data for The Prepared Dispersions

Formula	EE %*	Formula	EE %*	Formula	EE %*
F1	72.56 ±1.07%	**F10**	75.5 ±3.03%	**F19**	79.45 ±5.06%
F2	65.44 ±2.05%	**F11**	68.34 ±4.08%	**F20**	73.22 ±2.07%
F3	62.1 ±3.05%	**F12**	63.18 ±1.02%	**F21**	68.85 ±1.02%
F4	80.5 ±5.02%	**F13**	80.5 ±8.08%	**F22**	85.23 ±5.03%
F5	76.71 ±4.04%	**F14**	72.15 ±3.02%	**F23**	74.25 ±3.02%
F6	71.85 ±5.04%	**F15**	68.88 ±4.03%	**F24**	68.1 ±1.08%
F7	82.65 ±2.07%	**F16**	83.45 ±2.04%	**F25**	88.24 ±4.04%
F8	72.15 ±5.02%	**F17**	76.7 ±7.05%	**F26**	78.18 ±2.07%
F9	68.9 ±1.09%	**F18**	65.2 ±3.05%	**F27**	72.9 ±3.08%

*Mean ± SD, n=3

Study of vesicle size, polydispersity index (PDI), and zeta potential (ζ)

Optimized dispersions with optimum entrapment efficiency (≥80%) were evaluated for vesicle size, polydispersity (PDI), and zeta potential (ζ) measurements. Results are shown in Figure. 3 in which the type of terpene used was studied to predict its effect on the vesicle size of the prepared invasomes vesicles.

Results showed that the use of β-Citronellol as in (F4 and F7) had (385.8 ±8.4 nm and 443.1 ±5.7 nm) vesicle size, respectively which was higher than other optimized dispersions. The large vesicle size was related to the molecular weight of the terpene used in which it was agreed with obtained results that β-Citronellol had a higher molecular weight than the other used terpenes (M. wt. 156.27 g/mol). Other results showed that vesicles containing α-Pinene (M.wt 136.23 g/mol) as in (F13 and F16) showed (337.8 ±7.8 nm and 341.2±6.5 nm) vesicle size, respectively while D-Limonene containing vesicles (M.wt 136.24 g/mol) as in (F22, F25), showed (202.9 ±6.5 nm and 317.7 ±4.1 nm) vesicle size respectively. Statistical analysis revealed that the type of terpene used had a statistically significant effect (p<0.05) on vesicle size [10].

For the study of the effect of increasing the % of terpene used regardless of its type, data showed that as the % of terpene in the invasomes vesicles for the optimized dispersions increased from (4% to 8% w/v), the size of the vesicles expanded as shown in (F4, F13, and F22) which had (385.8 ±8.4 nm, 337.8 ±7.8 nm, and 202.9 ±6.5 nm), respectively when compared to (F7, F16, and F25) which had (443.1 ±5.7 nm, 341.2 ±6.5 nm, and 317.7 ±4.1 nm), respectively. Results revealed no significant difference (p>0.05) among dispersions in response to the effect of increased terpene % on vesicle size. Despite that, all optimized dispersions were in the nano size range and this could be attributed to the use of probe sonication during the method of preparation. The use of a probe sonicator might reduce the dispersion vesicle size as the time of sonication was optimized. The method of preparation was considered highly reproducible with the formation of vesicles in a nano size range which minimizes the cost and time in preparation. All dispersions tested for vesicle size measurement were accepted for further study. All tested dispersions were monodispersed in which the polydispersity index (PDI) was less than 1 (PDI<1) as the polydispersion decreased less than 1, the system is more uniform in vesicle size distribution and fewer variations in size were obtained. [32]. Concerning zeta potential (ζ), results showed that zeta potential values ranged from (-11.21 ±2.7 mV) for F7 to (-33.81 ±2.1mV) for F22 as shown in Figure. 4. Data showed a statistically significant difference (p<0.05) in zeta potential values among optimized dispersions. The incorporation of ethanol as a component in the invasomes vesicles made the vesicles acquire a negative charge, which accounts for the negative value of zeta potential. The dispersion might become more stable during storage against flocculation and phase separation as the zeta potential approaches (±30 mV) [33]. In general, a negatively charged vesicular system promotes efficient drug release from the vesicles. According to previous research, negatively charged vesicles release drugs more quickly than positively charged vesicles. This is explained by the fact that vesicles with positive charge molecules may act as a counter ion that form ion pairs with negatively charged drug molecules retarded their release [34]. In addition, the existence of a positive charge on the vesicle's surface has been reported to alter the drug permeation across the skin tissue. The skin had the potential to act as a negatively charged membrane [35]. The permeation of ONDS across the superficial skin stratum corneum can be enhanced by negatively charged vesicles since they typically exhibit a higher flux than their positively charged counterparts [36].

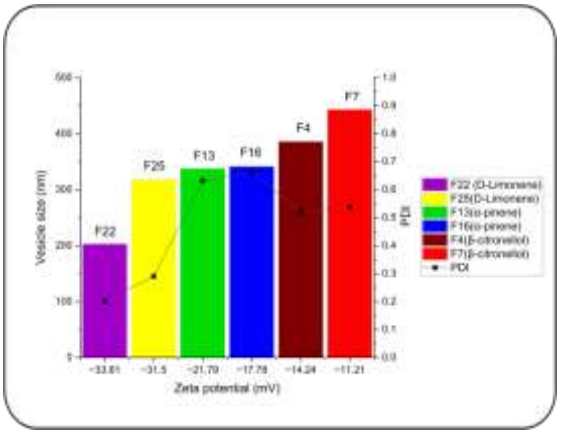
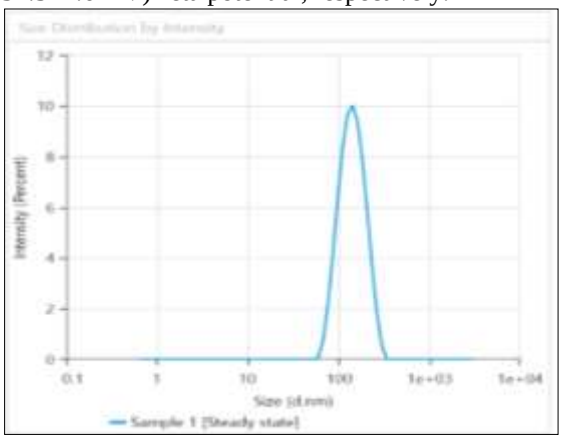


Figure 4. Vesicles size, PDI, and zeta potential for the optimized dispersions, n=3

Figure. 5 shows the graphical representation of vesicle size distribution and zeta potential value for the optimized dispersion (F25), which had (317.7±4.1 nm) vesicle size, (0.29±0.05) PDI and (-31.5±1.6 mV) zeta potential, respectively.



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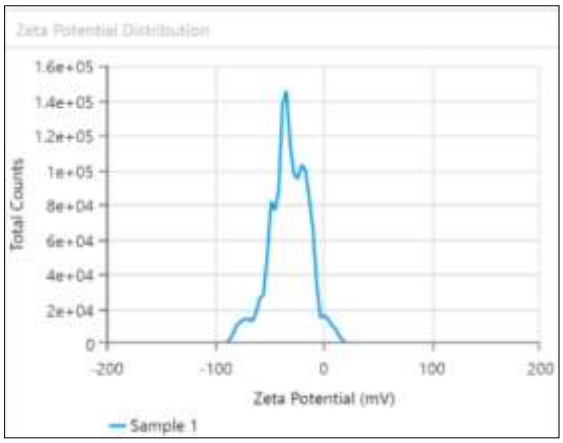

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Figure 5. Results for (F25), A) Vesicle size distribution, B) Zeta potential, n=3.

The in vitro ONDS dissolution study

Optimized ONDS loaded invasomes dispersions were evaluated for *in vitro* dissolution.

Results showed that terpene type affects the release behavior of ONDS from invasomes vesicles. Vesicles containing D-Limonene as in (F22 and F25) showed a higher % of cumulative ONDS release (69.8 ±1.5% and 75 ±2.5%) ONDS release after 12 h. respectively. While dispersions containing other used terpenes showed a lower % of ONDS release after 12h. as shown in (F4 and F7) which contained β-Citronellol showed (43.1 ±2.3% and 36 ±1.7%) ONDS release after 12 h. respectively and (F13 and F16) which contain α-Pinene showed (60.2 ±1.8% and 48.1 ±2.9%) ONDS release after 12 h. respectively. As a result, data showed a statistically significant difference (p<0.05) among dispersions relating to the type of terpene used on ONDS release from vesicles.

These results might relate to the solubilization efficiency of D-Limonene. Also, the presence of lecithins in an optimum amount act as a vesicle former component and as a surfactant ensuring ONDS in a solubilized form and infer more stable vesicles which avoids premature release of ONDS to the dissolution medium.

The low boiling point of D-Limonene (176ºC) and α-Pinene (156.2ºC) compared to β-Citronellol (224.5ºC) led to weak cohesiveness or self-association of the molecules, which led to a high % ONDS released [27]. As the boiling point of terpene increases, the cohesive forces of attraction or self-association between molecules become stronger, and the lower is the amount of ONDS released associated with a longer time. [29].

A less percent of cumulative ONDS release after 12h. was seen in (F4 and F7 and F13) in which these dispersions delay the release of ONDS for a longer time that exceed the daily dose intended to formulate [14].

Percent of terpene used might alter the release behavior of ONDS from invasomes vesicles. Results showed that dispersions with a high percent of terpene (8% v/v) as in (F7, F16, and F25) had a higher % cumulative ONDS release (43±2%, 60±2.7%, and 75±2.5%) after 12 h. when compared to other dispersions contained lower percent of terpene (4% v/v) as in (F4, F13, and F22).

Data showed that all optimized dispersions exhibited an extended-release behavior, as shown in Figure. 6.

Results showed a significant difference (p<0.05) in the effect of increase % of terpene used on ONDS release and this was also related to the solubilization efficiency of the terpene used in which as the terpene percent increases, more ONDS moved from the vesicles to the dissolution medium [10].

Analysis of *in vitro* dissolution profile data and comparison of the results among optimized dispersions where done by using the DDSolver® add-ins program by studying the similarity factor (f_2) of the dissolution curves for the optimized dispersions regarding the higher % release dispersion (F25). Data showed that (f_2) was equal to (48.06 ±3.44) and as a result, no similarity in ONDS release was observed as (f_2) should be more than 50% to consider the dissolution profiles similar. As a result (F25) was not like other dispersions and it was evaluated for kinetic modeling. Results showed that dissolution follows the Korsmeyer-Peppas model (R^2=0.99) as shown in Table. 3, with an anomalous release behavior from the vesicles (non-fickian) as (n) which identifies the mechanism of release showed it had (n=0.8 ±0.02) which was within the range (0.45 < n < 0.89) and this was agreed with the reported results from previous works [37].

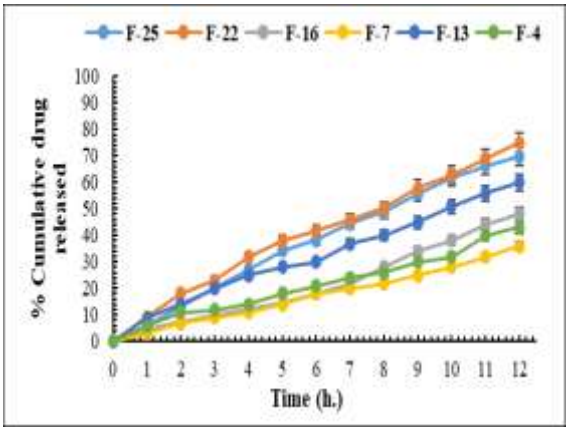


Figure 6. The *in vitro* cumulative drug release profiles for optimized dispersions using PBS (pH 7.4) at 37ºC for 12h. Error bars = SD, n=3.

Table 3. Dissolution Data Modelling for (F25), n=3

Formula	Zero-order		First-order		
	K_o	R^2	K_1	R^2	
F25	6.53 ±0.8	0.98 ±0.1	0.1 ±0.02	0.98 ±0.15	
	Higuchi		Korsmeyer-Peppas		
	K_H	R^2	K_{KP}	R^2	n
	17.41 ±1.8	0.89 ±0.11	9.8 ±0.7	0.99 ±0.08	0.8 ±0.02

The ex vivo ONDS permeation study

The HPLC analysis method developed was valid, accurate, reproducible, and sensitive for determining ONDS. It was separated as a single clear peak at (3 ±0.04 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 (R^2) reached unity (R^2= 0.99). The average percent recovery of ONDS was (98.25 ± 0.48%). Results related to the effect of type of terpene on the permeability of invasomes vesicles across rat skin membrane showed that flux (J_{ss}) values after 9h. time period for (F4 and F7) which contain β-Citronellol were (157.8 ±10.8 and 207.5±10.3) µg/cm^2. h. For α-Pinene containing vesicles as in (F13 and F16) showed (156.2 ±7.1 and 224.3 ±16.2) µg/cm^2. h. flux values, respectively. While (F22 and F25) were showed (272.6 ±12.5 and 340.3 ±14.7) µg/cm^2. h., respectively in which these dispersions contain D-Limonene. As illustrated in Figure. 7, data showed a significant effect (p<0.05) of D-Limonene containing invasomes vesicles when compared to other dispersions. The high (J_{ss}) values of dispersions containing D-Limonene were due to the high partition coefficient and the low boiling point of D-Limonene in which invasomes would be associated with the skin layer strongly and efficiently and as a result permeation efficiency enhanced. D-Limonene containing vesicles showed a higher permeation rate of vesicles across the rat skin membrane with time [38].

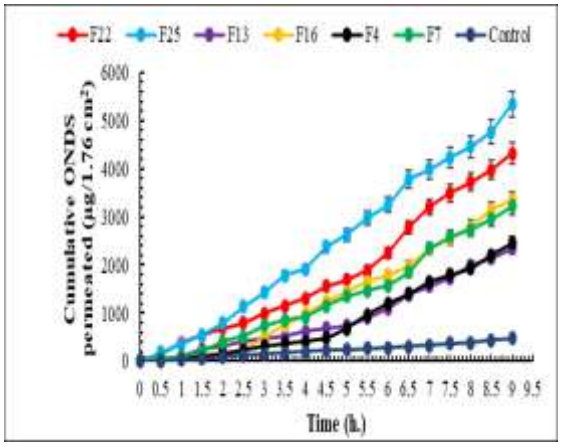


Figure 7. The *ex vivo* permeation of the optimized dispersions across rat skin membrane (1.76 cm^2) using Franz diffusion cell at (32±1 ºC). Error bars = SD, n=3

The permeation was remarkably enhanced by 11.5 times for (F25) when compared to plain (control) ONDS in PBS (10 mg/ml) in which the (J_{ss}) across rat skin was (340.3±14.7) µg/cm^2.h. for (F25) and (29.6 ±2.1 µg/cm^2.h.) for the control, respectively. Data is shown in Table. 5 represents the permeation parameters for the optimized dispersions. The effect of increasing % of terpene on the permeability was also studied. Results showed that the increase in the % of terpene from (4% v/v) as in (F22) to (8% v/v) as in (F25) which contained D-Limonene, the permeability increased significantly (p<0.05).

The differences in the permeation enhancement activity of terpenes at lower and higher percentages may be attributed to possible differences in the thermodynamic activity of terpenes in the vesicles [39]. The same scenario was also observed for (F4 and F7) which contained β-Citronellol and for (F13 and F16) which contained α-Pinene, respectively. Permeation of the dispersion in rat skin is expected to be higher than in human skin due to variations in the stratum corneum structure [20]. It was shown that D-Limonene in addition to ethanol and lecithin mixture may be considered an efficient permeation enhancer as they act synergistically to facilitate the permeation of ONDS across rat skin membrane [40]. All optimized dispersions were in the nano-size range as noted previously in which the nano invasomes might be responsible for the enhancement in permeation rate [41]. The desired flux (J_{ss}) for (F25) dispersion was higher than the target flux calculated from equation 2 (316 µg/cm^2.h.) so it can be considered as the best dispersion among others as it will exert an effective therapeutic effect of ONDS if it was formulated for dermal application. In addition, the lag time showed a delay in release and the optimized dispersion had variable lag times that affected (J_{ss}). The dispersion (F25) exhibited a significant (p<0.05) shorter T_{lag} (0.46 ±0.1 h.) as it illustrated by the higher (J_{ss}) than other dispersions According to equation 5, using the desired flux value of the selected dispersion (F25), the area needed for topical application in human for one daily dose is equal to 5.5 cm^2. The area of application is essential for formulations that are intended to be used as a transdermal formulation like patches, gels and microneedles. According to the desired flux value of (F25), the area of application should be equal to 5.5 cm^2 to give the desired therapeutic effect of ONDS. Accordingly, the daily dose for transdermal ONDS for human use ≈ 45 mg which is nearly equivalent to 5 ml of the selected (F25) invasomes dispersion (as ONDS EE% =88.24 ±4.04%). The selected dispersion was used later for the preparation of transdermal gel in a concentration that ensure an effective therapeutic effect of ONDS. The permeation flux of the dispersion will give correlation and prediction about the permeation flux of the final prepared gel from the selected ONDS-loaded invasomes dispersion (F25) [19].

Table 5. The Ex vivo Permeation Parameters for the Optimized Dispersions Across Rat Skin Membrane Compared to Control

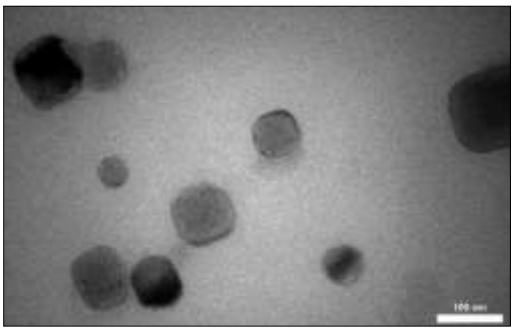
*Formula	Slope (dQ/dt)/ (1.76 cm^2)	(J_{ss}) µg/ (cm^2. h.)	Q_{9h} (µg) (1.76 cm^2)	Tlag (h.)	ER
F4	277.8 ±19.4	157.8 ±10.8	2460 ±122	1.39 ±0.3	5.3 ±0.9
F7	365.2 ±15.7	207.5 ±10.3	3215 ±160.75	0.9 ±0.2	7 ±1.2
F13	274.9 ±14.9	156.2 ±7.1	2500 ±125	0.7 ±0.3	5.3 ±1.8
F16	394.7 ±17.4	224.3 ±16.2	3355 ±167.75	1.14 ±0.2	7.6 ±0.5
F22	479.9 ±21.9	272.6 ±12.5	4320 ±216	0.67 ±0.2	9.2 ±0.5
F25	598.8 ±11.0	340.3 ±14.7	5340 ±267	0.46 ±0.1	11.5 ±0.7
****Control**	52.1 ±5.7	29.6 ±2.1	478 ±42.4	0.3 ±0.1	…..

*Mean ± SD

** Ondansetron in PBS (1% w/v)

Visualization of ONDS loaded invasomes vesicles by TEM

The TEM images were taken to visualize the shape and vesicle size for the selected dispersion (F25) in which results showed nano-sized vesicles with spherical or deformed shapes at different magnification power, as shown in Figure. 8. These results agree with the results obtained from zeta sizer measurements [24].



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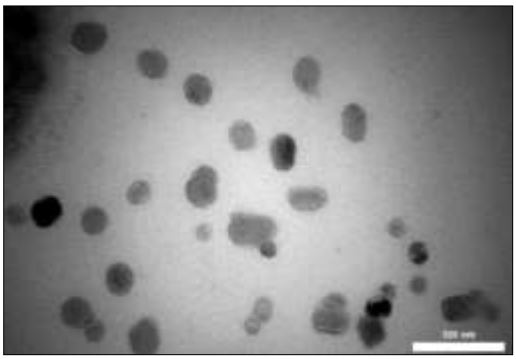

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Figure 8. The TEM images for optimized dispersion (F25) at 100nm scale (Magnification 140000X) and (B) at 300 nm scale (Magnification 60000X).

Conclusion

Ondansetron loaded invasomes dispersions could be prepared in an efficient and reproducible manner by the mechanical dispersion method. Prepared 1% (w/v) dispersion (F25) which contained D-Limonene (8% v/v), lecithin's mixture (2:2) (% w/v) and ethanol (20% v/v) showed optimized and desirable results than other dispersions. Results obtained from the *in vitro* dissolution and the *ex vivo* permeation showed that ONDS dissolution and permeation were enhanced by using a low percent of lecithin mixture (Egg: Soybean) (1:1) and a high percent of D-Limonene (8% v/v). Invasomes vesicles could be considered a promising drug delivery system for the transdermal route for ONDS. The study concluded that the ingredients of the prepared dispersions would facilitate the release, and permeation, improve therapeutic efficiency, and ensure one a daily dose requirement for ONDS application.

Future work

The selected dispersion (F25), which had optimized for efficient *ex vivo* permeation across rat skin, would be formulated as transdermal ONDS-loaded invasomes gel. Transdermal invasomes gel will be prepared and evaluated by *in vitro* and *ex vivo* studies. In addition, an *in vivo* animal study will be performed to measure and evaluate the pharmacokinetics parameters of ONDS for the transdermal route using the optimized invasomes gel and compare it to the oral route (marketed product) by measuring the relative bioavailability.

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Conflicts of Interest

The authors declare that they have no conflicts of interest related to this work.

Funding

None.

Ethics Statements

The ethical committee at the College of Pharmacy/University of Baghdad reviewed and approved all the procedures performed on animals by following the standard protocols for the care and use of laboratory animals.

The animal study followed the WOAH (Formerly Office International des Epizooties) foundation for animal ethical principles in studies involving animals and animal specimens.

The animal study complied with the ethics as reported in the guidelines written by the National Committee for Research Ethics in Science and Technology (NENT), Norway. The ethical approval code is (REAFUBCP11210204A) registered on 01-12-2021.

Author Contribution

Study conception and design: Omar S.; data collection: Omar S.; analysis and interpretation of results: Omar S. and Entidhar J.; draft manuscript preparation: Omar S. and Entidhar J. All authors reviewed the results and approved the final version of the manuscript.

Supplementary materials

1. Available link for ethical approval sheet: https://drive.google.com/file/d/1rf0mdkKA3vQH LM1c9bkAC0_NKMaO0iKc/view?usp=sharing
2. Available link for DDSolver® add-ins program: https://drive.google.com/file/d/ 1L2A LVs 2Cx-MC6Z_KO23e_Q0s3vx_siuR/view?usp=sharing

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