Nanoemulsion-based nasal *in situ* gel of olanzapine.

Zahraa Hussein Ali[a], Myasar Alkotaji[b*]

[a]College of pharmacy, University of Mosul, Mosul, Iraq
[b]College of pharmacy, Ninevah University, Aljawseq, Mosul, Iraq

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ABSTRACT

Oral delivery of olanzapine suffers from low oral bioavailability and there has been reports of metabolic side effects. This study aimed to prepare a nanoemulsion of olanzapine and incorporate it into an *in situ* gel for nasal delivery. Such nasal delivery of olanzapine could provide prolonged contact time with the nasal mucosa and a potential enhanced action with lower side effects. Several formulations of nanoemulsions were prepared and characterized through the measurements of conductivity, transmittance, pH, viscosity, hydrodynamic diameter, polydispersity index, Zeta potential, entrapment efficiency, and release profiles. In addition, a pharmacodynamics study was conducted through animal studies. The selected formulation showed excellent nanoemulsion with a size in the nanometre range, a good polydispersity index with acceptable stability as indicated by the thermodynamic stability test. The cumulative percentage of olanzapine released from the nanoemulsion showed a good release profile. Pharmacodynamics study on rats using Paw test demonstrated a very clear enhancement in the antipsychotic efficacy of olanzapine in the following order: Nanoemulsion-based *in situ* gel (with HPMC)>nanoemulsion-based *in situ* gel >nanoemulsion>solution. Interestingly, olanzapine from the nanoemulsion-based *in situ* gel showed comparable antipsychotic efficacy to haloperidol, when given intraperitoneally. Nanoemulsion-based nasal *in situ* gel is a promising drug delivery system for olanzapine for achieving a targeted delivery. However, further investigations on olanzapine accumulation in the brain after such delivery are recommended.

KEY WORDS: Nanoemulsion, nasal *in situ* gel, nanoemulsion-based *in situ* gel, olanzapine, nasal delivery

INTRODUCTION

Schizophrenia is a common psychiatric diseases, affecting around 1% of the population (1, 2). It is characterized by a wide number of symptoms that affects perceptions, thoughts, cognition, and emotions (2).

The majority of currently available antipsychotic drugs are administered either by intramuscular injection or orally (3). However, there are many drawbacks to these delivery routes making them impractical and unreliable in some situations (4). For instance, the parenteral route is invasive and requires a certain level of skill making it impractical for self-administration (3). Furthermore, parenteral formulations may be inapplicable to anti-psychotic drugs as many of these medications cannot fulfill the requirements for these dosage forms such as having a good aqueous solubility or there may be safety concerns (4).

*Corresponding address: College of pharmacy, Ninevah University, Aljawseq, Mosul, Iraq, E-Mail:

On the other hand, the oral route has its own limitations. Firstly, poor systemic bioavailability caused by either enzymatic degradation or hepatic first-pass metabolism thus requiring higher doses to be taken to be effective. Also, a patient may be unconscious or have difficulty swallowing which may interfere with their adherence to the therapeutic program (3).

Olanzapine is an effective, atypical antipsychotic drug used for the treatment of schizophrenia. However, more than 40% of the orally taken olanzapine undergoes first-pass metabolism in the liver (5). In addition, it has been shown that the oral administration of olanzapine conventional tablets is associated with a higher risk of metabolic side effects, such as weight gain resulting in obesity, when compared to the administration of the same medication in alternative dosage forms such as orally disintegrating tablets or depot form (6, 7) This phenomenon was first reported in 2004 by De Haan *et. al.,* who showed that the replacement of a conventional tablet of olanzapine by an orally disintegrating tablet of olanzapine had resulted in weight loss in patients diagnosed with schizophrenia (8). This indicated an involvement of peripheral mechanisms controlling the side effects with little central involvement (9). Thus there has been an increasing focus on scientific research toward the development of an olanzapine dosage form that could be administered by alternative routes (10).

One promising route of administration is via the nasal passage which is considered to be a very attractive way of delivering antipsychotic drugs (10). The nasal route has been recognized for many years as suitable for the delivery of many drugs intended to have either a local or systemic effect (10). There are numerous reasons for considering this route as an effective systemic delivery of neuroleptic therapies including the large absorption area provided by the presence of microvilli at the epithelial surface, the rich blood supply of the sub-epithelial layer and the loose endothelial membrane (10, 11). The intranasal administration of the drug is associated with avoidance of first-pass metabolism (12). It results in rapid onset of a pharmacological effect, which is obtained by administering smaller doses of the drug (12). In addition, nasal administration can be considered for self-administration (10, 12). Furthermore, there is a great possibility for the delivery of medications administered intra-nasally through the olfactory nerves directly to the brain and thus circumventing the blood-brain barrier (BBB) (12, 13). This pathway is very promising for the delivery of neuroleptic drugs since the BBB is the most significant limitation for the reach of these medications to their target site in the brain (14).

Different particulate systems have been used for nasal to brain delivery including nanoemulsions (NEs). NEs are biphasic liquid dispersions of two immiscible liquids forming either oil in water or water in oil emulsion stabilized by a suitable blend of emulsifying agents (surfactants and co-surfactants) with a droplet diameter in the range of 100 nm (15). This small droplet size prevents the destabilization mechanisms including sedimentation, coalescence and creaming resulting in prolonged physical stability (15). NEs could be incorporated into various types of formulations such as liquids, sprays, creams, ointments, gels and others which could be delivered by different routes. They offer an excellent solution for stability or solubility problems that accompany many drugs (15). For instance, hydrophobic drugs could be dissolved in the oil phase and after administration, the drug will be released from oil droplets into the surrounding aqueous environment where nanoprecipitation take place. These extremely small-sized precipitates provide the extensively large surface area with greatly enhanced dissolution rate and improved bioavailability (15). Concerning stability, NEs can protect the drug (by encapsulating it within oil droplets) from enzymatic degradation, hydrolysis, oxidation and other instability issues under physiological circumstances (15). Therefore, NEs provide a very promising dosage form for delivering olanzapine intranasally.

The most important drawback to the nasal delivery of NEs is the limited nasal residence time (16). Numerous strategies have been proposed to prolong the contact time. One of them is using *in situ* gels (17, 18). These formulations maintain their liquid form outside the body and the gelation occurs once it comes into contact with mucous membranes (16, 19). Different triggering

factors can induce the gelation including thermal stimuli, pH, ionic strength or a combination of them (17). These dosage forms preserve the advantage of being liquid during administration leading to precise and easy intake along with prolonged nasal retention because of the limited nasal clearance (16).

In this study, different formulations of nano-emulsions were prepared and incorporated into the *in situ* gel. Poloxamer 407 was used as a thermosensitive *in situ* gelling agent along with hydroxypropyl methylcellulose as a mucoadhesive. The prepared NEs were fully characterized and the *in situ* gel was evaluated by an *ex vivo* permeation study and an *in-vivo* pharmacodynamic test.

MATERIALS AND METHODS

Drugs and reagents

Olanzapine was received as a gift from the Al-Kindi company for pharmaceutical industries (Iraq). (Z)-octadec-9-enoic acid (oleic acid) was purchased from the GC chemicals corporation (Spain). Polyoxyethylene (20) sorbitan monooleate (tween 80) was bought from the Scharlab corporation (Spain). Polyethylene glycol 400 was purchased from Fluka Riedel-DeHaen company (Switzerland). Poloxamer 407 was obtained from Sigma-Aldrich company (USA). Hydroxypropyl methylcellulose (HPMC) grade K4M was purchased from HiMedia lab (India).

THE DEVELOPMENT AND OPTIMIZATION OF OLANZAPINE LOADED NANO FORMULATIONS

The construction of the pseudo ternary phase diagrams

In order to determine the optimal concentration of the oil, surfactant, co-surfactant and water at which a nanoemulsion would exist, pseudo ternary phase diagrams were developed at an ambient temperature using the aqueous titration method (20). A mixture of surfactant and co-surfactant was blended at a specific volume ratio (1:1, 2:1 and 1:2) and termed Smix. Different phase diagrams were plotted. For each phase diagram, oil and Smix were mixed thoroughly in different volume ratios until all the possible ratios of oil and Smix were produced. Seventeen different combinations of oil and Smix were prepared (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0.8:9.2, 0.6:9.4, 1:8, 1:7, 1:6, 1:5, 1:3 and 1:2). To these combinations, water was slowly added dropwise and turbidity was observed visually. The quantity of water at which phase transition from transparent to turbid took place (and, sometimes, back to transparent once again) was recorded to delineate the boundaries of each phase accurately (20). CHEMIX school software was used to process the data and to construct the pseudo ternary phase diagrams (20).

Preparation of olanzapine loaded nanoemulsion

With the aid of the previously constructed pseudo ternary phase diagrams, eight different formulations were prepared.

Olanzapine loaded nanoemulsions (OnanoE) were prepared by self-nano emulsifying method. They were then stabilized using a high-speed homogenization technique. For this method, oleic acid (as the oil phase), Tween 80 and polyethylene glycol 400 (as surfactant and co-surfactant, respectively) were mixed together. Then the required amount of olanzapine was added and dissolved in this mixture with the aid of a sonicator (Power Sonic 410 Copley Scientific, U.K). Once a clear solution was obtained, the predetermined volume of water was added drop wise continuously mixing at ≈350 RPM (at room temperature) using a magnetic stirrer (Fisher Scientific, Korea). The prepared nanoemulsions were then homogenised at 25000 RPM using a high-speed (rotor-stator) homogeniser (Power Gen 125® Fisher Scientific, Germany) for three cycles of five minutes each with five minutes off between them. The composition of each formulation is provided in Table 1.

Preparation of nanoemulsion based nasal *in situ* gel (OnanoEG)

Olanzapine loaded nanoemulsion based *in situ* gels were prepared by cold method. For this method, the required weight of poloxamer 407 was added and dissolved in 2 ml of cold water (4-5°C) with the aid of an ice bath. The blend was left in the fridge

Table 1 The composition of the different formulations of olanzapine nanoemulsions

FORMULATION	OLANZAPINE (mg/ml)	(SMIX) TWEEN 80 TO PEG 400 RATIO	SMIX % (v/v)	OLEIC ACID % (v/v)	DISTILLED WATER % (v/v)
D1	8.5	2:1	30	4	66
D2	8.5	2:1	35	4	61
D3	8.5	2:1	40	4	56
D4	8.5	1:1	30	4	66
D5	8.5	1:1	35	4	61
D6	8.5	1:1	40	4	66
D7	8.5	2:1	30	6	64
D8	8.5	1:1	30	6	64

overnight to allow for the complete hydration of the polymer. Then, eight ml of the optimized olanzapine nanoemulsion were cooled to 4-5°C and combined with the cold polymer solution so that the resulting preparation had the required percentage of poloxamer 407.

For the addition of HPMC into the formulation, it was first dissolved in the cold water before the addition of poloxamer 407. The compositions of the prepared *in situ* gels are presented in Table 2.

Evaluation of the prepared olanzapine nanoemulsions

Droplet size and zeta potential measurements

Litesizer 500 (Particle size analyzer, Anton Paar, Austria) was used to determine droplet size (along with the polydispersity index (PDI)) and zeta potential of the formulated nanoemulsions through the application of dynamic light scattering (DLS) and electrophoretic light scattering technologies, respectively (21). The samples were first diluted with distilled water at the ratio of 1:2 (nanoemulsion:water) to exclude the effect of overlapped scattering. The droplet size was given as a mean hydrodynamic diameter. Kalliope™ software (version 2.8.3) was used to analyse the data. All the measurements were carried out in triplicate.

Entrapment efficiency determination

The entrapment efficiency (EE) was calculated by a direct method in which the amount of the drug that had been encapsulated within the droplets was measured (22). This was investigated by filtering the samples through a 0.2 μm syringe filter to eliminate any un-encapsulated drug (23). The filtrate was then diluted with methanol (1/1000 (v/v)) and the concentration of olanzapine was determined by UV-visible spectrophotometer (Labomed UVD-3000, USA). Finally, the EE% of olanzapine was calculated using Equation 1.

$$EE\% = \frac{\text{the quantity of olanzapine entrapped}}{\text{the total quantity of olanzapine added}} = x\ 100 \qquad \text{Eq. 1}$$

All the calculations were carried out in triplicate.

Turbidity transmittance

The turbidity of the prepared nanoemulsions was measured through the determination of the transmittance percentage (T%) of the samples after a dilution of 1 to 10 with (distilled water) DW using a

Table 2 The composition of the prepared *in situ* nasal gel formulations

FORMULATION	OLANZAPINE (mg/ml)	OLEIC ACID % (v/v)	SMIX % (v/v), RATIO	POLOXAMER 407 % (w/v)	HPMC % (w/v)	DISTILLED WATER (ml)
$D4_A$	6.8	3.2	24, (1:1)	16	-----	Up to 100
$D4_B$	6.8	3.2	24, (1:1)	16	0.6	Up to 100

UV-visible spectrophotometer (Labomed UVD-3000, USA) at a wavelength of 630 nm (24). Distilled water was used as a blank. All the readings were carried out in triplicate to calculate the mean values.

pH and conductivity measurements

The prepared nanoemulsions were first diluted 1 in 10 with distilled water and then the pH and conductivity were measured (at room temperature) using a calibrated digital pH meter (Eco Tester pH2®, Eutech, India) and a conductivity instrument (Senz μSiemen digital conductivity tester, Indonesia), respectively (25). All the measurements were carried out in triplicates and the mean values were calculated.

Viscosity measurement

Viscosity measurements were performed with aid of a cone and plate viscometer (Brookfield DV2T, USA). All the readings were taken at room temperature (without dilution). The instrument was assessed to make measurements at 12 RPM (with the exception of D2 and D3 for which 2 RPM was used for the test since these two formulations were so thick that an error would occur if 12 RPM was to be utilized). All measurements were carried out in triplicate and mean values were calculated (26).

Nanoemulsion stability tests

The prepared nanoemulsions were subjected to three different thermodynamic stability tests (27). First, a heating/cooling test was performed where the prepared formulations were kept at two different temperatures (40°C and 4°C) for no less than 48 hours each and the process was repeated for six cycles.

The formulations that passed the heating/cooling test were further evaluated using a centrifugation test in which the samples were centrifuged (Labofuge A, Germany) at 3500 RPM for 30 minutes and then examined to exclude any cracking or phase separation (28).

Then, a freezing/thawing test was performed on the successful preparations. During this test, nanoemulsions were exposed to three cycles of two alternating temperatures (21°C and 0°C). They were maintained for a minimum 24 hours at each temperature.

In vitro drug release studies

The *in vitro* release profiles of olanzapine from nanoemulsions were evaluated by the application of the dialysis bags technique in a USP dissolution apparatus type II (Copely, UK). 200 ml Phosphate buffer saline (PBS) pH 6.4 was used as the release media (29). One millilitre of the investigated formulations (D4, D7 and D8) was carefully placed in the dialysis bag, previously soaked overnight in water, then sealed from the two ends (29, 30). The dissolution apparatus was set at 50 RPM and maintained at 37±0.5°C (29). At specific time intervals, aliquots of 5 ml were removed from the release media and replaced by a similar volume of fresh PBS every time. The withdrawn samples were then appropriately diluted and analysed at λmax (270 nm). The process was carried out in triplicate and mean values were calculated. The release profiles were obtained by plotting the percentage of cumulative drug release against time.

Different kinetic-controlled models (zero-order and first-order) and diffusion-controlled models (Higuchi model) were used to investigate the release kinetics of olanzapine from nanoemulsions by fitting the data of cumulative release into these models (31).

Evaluation of nanoemulsion-based nasal *in situ* gel

Solution-gel transition temperature determination

The selected nanoemulsion formula was D4, which was then processed to formulate two different *in situ* gel formulations (denoted as D4a and D4b). The only difference between these two formulations is the inclusion of HPMC polymer in D4b but not in D4a. The prepared *in situ* gel formulations were tested for their solution-gel transition temperature using the tube-tilting technique to determine the temperature at which gelation takes place (32). For this method, approximately 2 ml of the cooled formulation was placed into a test tube. The tube was placed in a

thermally controlled water bath at 4°C. Then, the temperature of the water bath was raised gradually in a controlled manner. The sol-gel transition occurred when the surface of the preparation would not bend with 90° tilting of the tube.

Ex vivo permeation test

This test was performed using a Franz diffusion cell (PermeGear, USA) through a sheep nasal mucosa, which was obtained freshly from a local slaughterhouse with the help of a specialist surgeon (33). The tissue was allowed to equilibrate in PBS pH 6.4 for 15 minutes. The receptor compartment of the diffusion cell was filled with 5 ml of the diffusion media (PBS pH 6.4) and placed inside a stainless-steel bowl containing warm water (at 34 ± 1°C). The membrane was then mounted between the donor and receptor compartments with the mucosal side facing the donor compartment and the diffusion surface of about 0.503 cm^2. The diffusion media was constantly stirred by a teflon-coated magnetic bar so that the surface of the nasal membrane was continuously flushed by the diffusion media. After that, the two compartments were clamped together. Samples (50 microliters) of either D4a or D4b were placed in the donor compartment. At predetermined time points, 0.3 ml of the samples were withdrawn from the receiver compartment, properly diluted with methanol and filtered through (0.45) μm filter in order to be analyzed spectrophotometrically. After each sample collection, an equal volume of fresh PBS was used to replace the withdrawn volume. The study was carried out in triplicate and mean values were calculated.

The data were analyzed in order to determine the permeability parameters of olanzapine (34). The flux (J, mg/cm^2.hr) was calculated by dividing the amount of the drug permeated (m, mg) by the surface area of the diffusion mucosa (A, cm^2) and the time length (t, hr) using Equation 2.

$$J = \frac{m}{A.t} \qquad \text{Eq. 2}$$

The permeability coefficient (Kp, cm/hr) was determined by dividing J over the initial concentration of the drug present in the donor compartment (Cd, mg/cm^3) using Equation 3.

$$Kp = \frac{J}{Cd} \qquad \text{Eq. 3}$$

In vivo pharmacodynamic study

The *in vivo* pharmacodynamic study (the paw test) is a well-recognized test used in many studies (35, 36). A wooden platform was utilized for the test. It has two holes of 5 cm diameter for the hindlimbs, two holes of 4 cm diameter for the forelimbs and a slit for the tail.

Swiss albino rats aged 10-12 weeks and weighing 225-250 g were used for the experiment. The animals were housed individually one day before the test. Each animal was used only once and the test was held at 09:00-11:00 a.m. The rats were divided into six groups with five animals each. Four groups received an intranasal application of either olanzapine solution (Osol), OnanoE, D4a or D4b in a dose of 0.64 mg/kg divided into two nostrils and delivered through the use of a micropipette attached to a thin plastic tubing with a 0.47 mm internal diameter. The fifth group was given 10 μl of normal saline in each nostril (negative control group) and the sixth group received 1mg/kg of haloperidol intraperitoneally (positive control group).

The test started 30 minutes after the drug had been administered by placing the rats over the platform and gently placing the hindlimbs in their holes followed by placing the forelimbs in their holes and finally the tail in its slit. The time required by the rat to withdraw either of its forelimbs (forelimb retraction time FRT) and hindlimbs (hindlimb retraction time HRT) was recorded. The test was repeated at 40 minutes and 50 minutes after administration and the mean FRT and HRT were calculated for each animal.

One second was considered as the minimum for both FRT and HRT while 30 seconds was taken as the maximum for both of them (37). The work on animals was authorized by the specialist committee at

the University of Mosul (Number UM.VET.2021.003).

An olanzapine solution was prepared for comparative purposes by dissolving 85 mg of olanzapine powder in a mixture of 8 ml of propylene glycol and 2 ml of ethanol so that the final strength of the solution is 8.5 mg/ml (38).

Stability test of the nasal in situ gel

Stability tests were carried out for the D4a and D4b formulations, to which an anti-oxidant (alpha-tocopherol, final concentration is 0.05% (v/v)) and a preservative (methylparaben, final concentration is 0.025% (w/v)) were incorporated (39). About ten ml of these two formulations were stored in tightly closed amber glass containers in the refrigerator. After three months, the samples were checked physically for any alterations in appearance, pH, conductivity and sol-gel transition temperature.

Statistical analysis

All the results of the experimental work were expressed as mean ± standard deviation. Unpaired t test and one-way analysis of variance (ANOVA) test (followed by Duncan test to check the significance of the ANOVA test) were employed for the statistical analysis since the continuous data were normally distributed. A p-value ≤ 0.05 was regarded as statistically significant. Minitab statistical software (version 19 Minitab, Ink) was utilized to assess the experimental data.

RESULTS AND DISCUSSION

Selection of materials

Various parameters influenced the selection of the excipients for the formulations of the nanoemulsions. Different oils were screened to find one suitable depending on reported safety and solubility data (40–43). Oleic acid meets most of the requirements for use as an oil phase, particularly the olanzapine solubility (which is 203.27mg/ml), in addition to being safe and biologically compatible. Therefore, it was selected to be the oil phase (43).

A blend of surfactant and co-surfactants should be incorporated (in order to secure the stability of the formed nanodroplets). The HLB value of the selected surfactant blend is another specification that should be taken into account in the selection. For the fabrication of o/w nanoemulsion, the HLB value of the surfactant mixture should be greater than 10 (43).

Thus polyoxyethylene sorbitan monooleate (Tween 80) (HLB value 15) and polyethylene glycol 400 (HLB value 11.4) were selected for use as a surfactant and co-surfactant, respectively. Both of these surfactants and co-surfactants met the demands for the preparation of o/w nanoemulsion (44, 45). In addition, the safety profiles of these two excipients are well established especially for PEG 400, which is considered nontoxic at the concentration used in such studies (39). On the other hand, tween 80 is an non-ionic surfactant and it is well known that cell toxicity of this type of surfactant is much lower than for the ionic type (46, 47). Accordingly, the selection of such materials would eliminate the need for performing cell toxicity studies. However, histopathological study was conducted on nasal tissue.

The construction of the pseudo ternary phase diagram

For the construction of the pseudo ternary phase diagrams, all the data collected during the aqueous titration procedure for each of the three different ratios of surfactant to co-surfactant (Smix) (1:1, 2:1 and 1:2) were mapped with the aid of CHEMIX school software and the resulting diagrams are shown in Figure 1.

The pseudo ternary phase diagrams are triangle shaped with each axis representing the oil, Smix or water. In these diagrams, the nanoemulsion zone was determined as the region where translucent or clear blends were produced during the aqueous dilution based on a visual inspection of the mixtures. The size of these areas, among the various diagrams, were compared since the diagram with the greater nanoemulsion zone has a favourable opportunity for forming stable nanoemulsions and avoiding the formation of metastable formulations (48, 49).

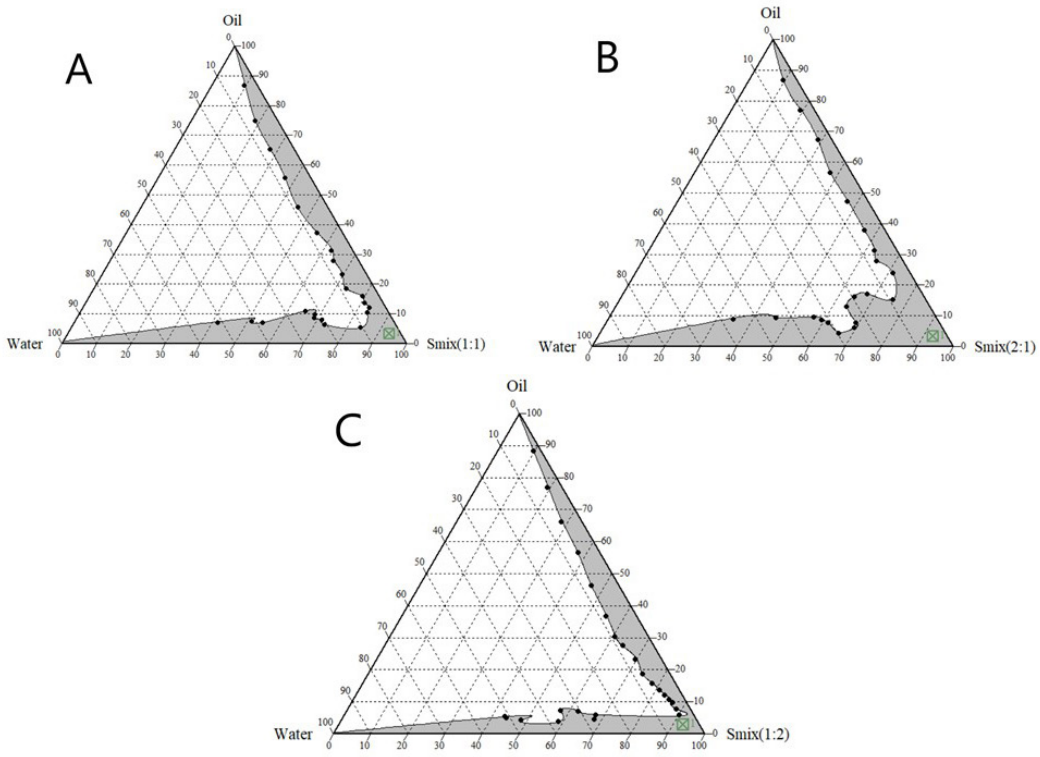

Figure 1 The pseudo ternary phase diagrams of different Smix systems. (A) is the diagram for the (1:1) Smix, (B) is for the (2:1) Smix and (C) is for the (1:2) Smix.

In each of these diagrams, the shaded grey area accounts for the nanoemulsion region while the unshaded area is consistent with the turbid (two phases or emulsion) region. The obtained pseudo ternary phase graphs demonstrated that the increment in the concentration of the Tween 80 (surfactant) with respect to PEG 400 (co-surfactant) had resulted in a larger nanoemulsion region.

The zone is largest in diagram B with (2:1) Smix ratio, slightly smaller in diagram A with (1:1) ratio and very small in diagram C with (1:2) ratio. These observations are similar to those reported previously in literature which used the same surfactant combinations (50). This phenomenon may be attributed to the higher ability of Tween 80 (because of its higher HLB value) to reduce the interfacial tension and to cause an increase in the interface fluidity (51). Accordingly, only (2:1) and (1:1) Smix ratios were considered in the preparation of the loaded nanoemulsion while the (1:2) Smix was neglected.

Evaluation of the prepared olanzapine nanoemulsions

The formulations exhibited a droplet size within the nano range. The droplet size plays a corner role in determining the rate and extent of drug release from the formulation. The lower the droplet size, the higher the permeation across the epithelial membranes and thus the greater the bioavailability of the drug (52). Polydispersity index, on the other hand, provides an indication of the uniformity of the droplet size distribution within the preparation. The smaller the polydispersity index, the greater would be the droplet size uniformity. The value of ≤ 0.3 is considered within an acceptable distribution of droplet sizes since multi dispersed systems are at greater risk of large globule growth resulting from Ostwald ripening that causes the small droplets to stick with the surface of the large globules forming energetically stabilized system (52). The results for hydrodynamic diameter measurements together with the polydispersity indices are provided in Table 3.

Table 3 The hydrodynamic diameter, polydispersity index, zeta potential and entrapment efficiency percentage of the prepared formulas of nanoemulsions. Results are expressed as mean ± SD (n = 3)

FORMULATION	SMIX %, RATIO	OIL %	HYDRO-DYNAMIC DIAMETER (nm)	POLYDISPERSITY INDEX (PDI)	ZETA POTENTIAL (mV)	EE%
D1	30 (2:1)	4	111±20	0.21±0.04	-5.4±0.1	92.6±2.15
D2	35 (2:1)	4	155±6	0.25±0.02	-8.4±0.1	84.41±9.37
D3	40 (2:1)	4	--------	--------	-------	-------
D4	30 (1:1)	4	111±3	0.20±0.01	-5.64±0.64	94.74±5.07
D5	35 (1:1)	4	129±9	0.22±0.02	-3.6±0	93.19±3.91
D6	40 (1:1)	4	125±40	0.22±0.01	-10.57±4.5	84.4±2.73
D7	30 (2:1)	6	98±1	0.25±0.00	-4.9±0	93.47±0.49
D8	30 (1:1)	6	194±7	0.26±0.01	-4.9±0.1	87.82±6.93

The droplet size distribution profile is another important indicator to distinguish between nanoemulsions and microemulsions (53). Microemulsions tend to display a single sharp peak on particle size distribution while nanoemulsions usually have a single wide peak or multiple peaks (53, 54). Figure 2 shows the droplet size distribution profiles for all the formulated nanoemulsions. All the formulations exhibited normal distribution patterns with multiple peaks (one of them was very tiny) with the exception of formulation D4 which demonstrated a single peak (mono-modal distribution). These results confirm that the dispersions are nanoemulsions rather than microemulsions (53).

Zeta potential provides an indication of the electrical charge at the droplet surface (55). Generally, for a formulation of a stable nanoemulsion, the droplets should possess an absolute value of zeta potential greater than 30 mV (with either a positive or negative charge) (56). Droplets with low absolute value tend to aggregate over time rendering them relatively unstable.

However, the Tween 80 stabilized nanoemulsions possess a relatively low negative zeta potential but they demonstrate good stability because their large polymeric heads provide sufficient steric repulsive forces (they are stabilized by steric forces rather than by electrostatic forces) (56, 57). Table 3 shows the measured zeta potentials of the prepared nanoemulsions.

Entrapment efficiency (EE) is an important indicator for the effectiveness of the drug within the formulation and its actual delivery to its target after administration (58). The results for EE% are provided in Table (3). However, the EE% of formulation D3 was very difficult to measure because of its high viscosity. This made it difficult to pass through the membrane filter. Although there are variations in the entrapment efficiency, all of these alterations were statistically insignificant (p-value ≤ 0.05).

All prepared nanoemulsions showed good conductivity which support the fact that these nanoemulsions are of the oil in water type (59). The reason for this conclusion is that oil in water emulsions (in which water is the external phase) usually demonstrates a conductivity value near 100 μS/cm (due to the high water conductance) while water in oil emulsions show a conductivity magnitude which is 100-1000 times smaller than that (59). The results for nanoemulsion conductance are presented in Table 4.

The transmittance percentage of the prepared nanoemulsions varied widely. There is a relationship which correlates the degree of turbidity of a nanoemulsion to the droplet size. However, this was not applicable in all situations in this study. For instance, the percentage of transmittance for formulations D2, D6 and D8 were very low while their droplet size measurements were within nanosize. This high turbidity may be attributed to the unentrapped drug particles (the entrapment efficiency was 84.41, 84.4 and 87.82 for formulations D2, D6 and D8, respectively) which remain suspended in the aqueous phase and because of their inherited yellow colour, they imply a hazy turbid appearance to the final product with low

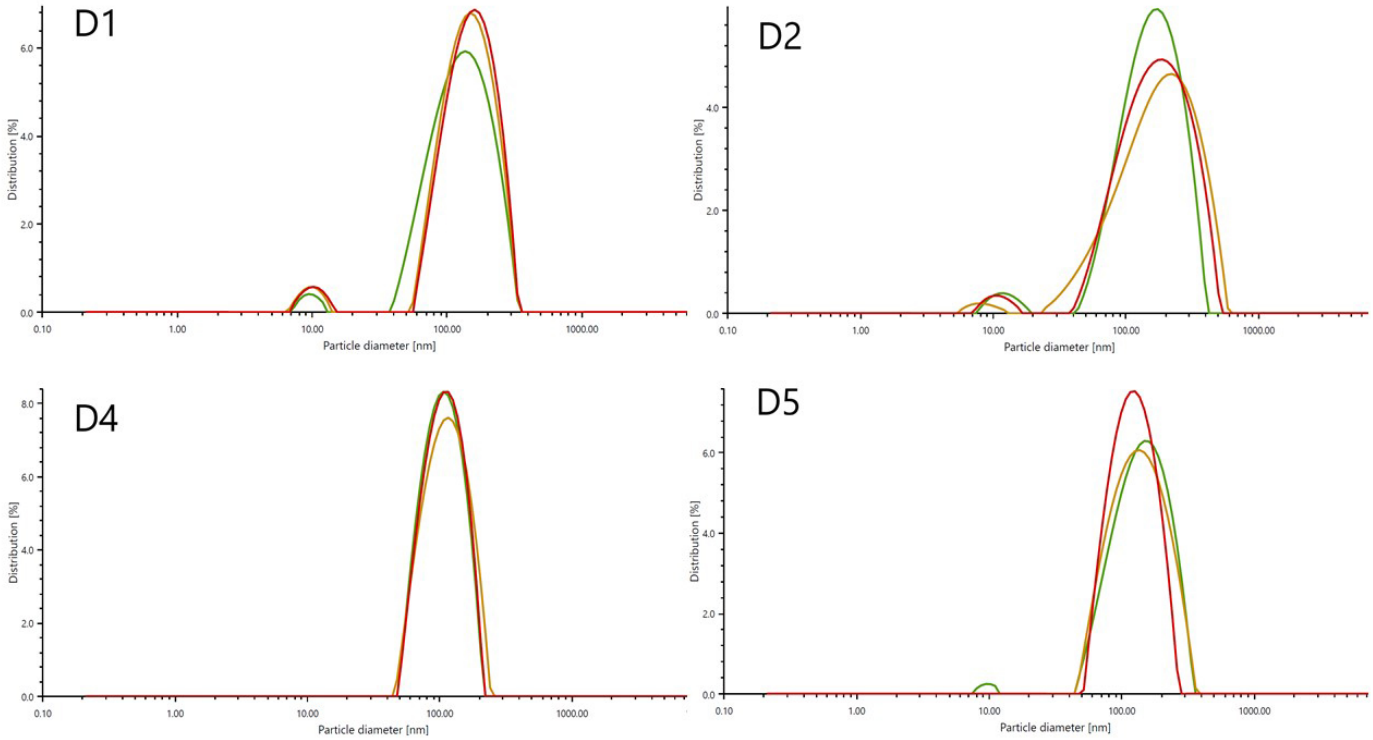

Figure 2 (A) The droplet size distribution in formulations D1, D2, D4 and D5.

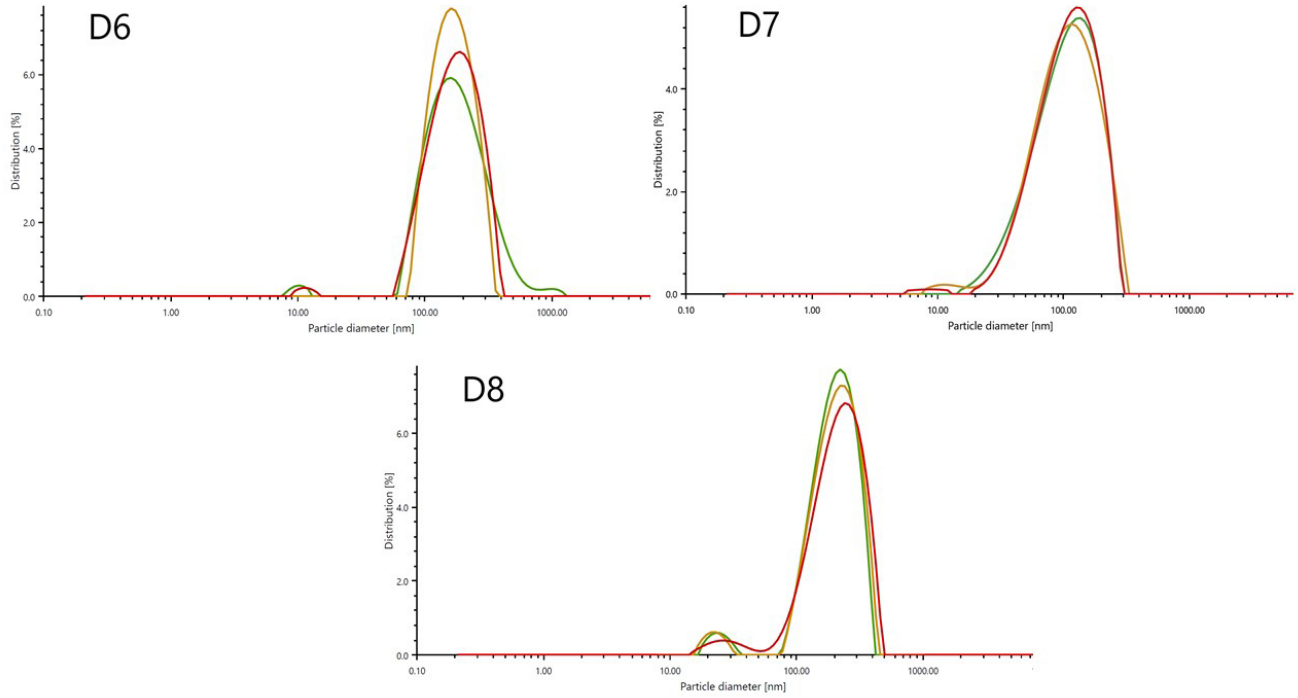

Figure 2 (B) The droplet size distribution in formulations D6, D7 and D8.

Table 4 The conductivity, transmittance percentage, pH and viscosity of the prepared nanoemulsions. Results are expressed as mean ± SD (n = 3)

FORMULATION	SMIX %, RATIO	OIL %	CONDUCTIVITY (μSiemen/cm)	TRANSMITTANCE %	PH	VISCOSITY (cP)
D1	30 (2:1)	4	107.5±10.5	83.19±4.12	6.3±0.3	19.34±0.197
D2	35 (2:1)	4	120.5±3.5	69.31±6.21	6.25±0.35	*243.3±13.25
D3	40 (2:1)	4	-------	36.48±5.0	6.6±0.1	*326.57±13.99
D4	30 (1:1)	4	121.5±3.5	73.06±2.1	6.45±0.05	7.68±0.06
D5	35 (1:1)	4	113±24	75.66±4.87	6.15±0.35	44.77±0.58
D6	40 (1:1)	4	126.5±2.5	38.05±17.68	6.15±0.35	57.37±0.34
D7	30 (2:1)	6	90±12	74.75±1.64	6.05±0.05	18.2±0.04
D8	30 (1:1)	6	93.5±9.5	37.8±2.48	6.0±0	30.29±0.78

*These viscosity results were obtained using a 2 RPM setting instead of the 12 RPM setting used for the rest of the formulations.

measured transmittance. The transmittance results were not considered for further evaluation.

The optimum pH of the prepared nanoemulsions depends mainly on two factors. First, the pH should be within the range of the pH of nasal secretion (which is between 5 and 6.5) in order to minimize the risk of irritation to nasal mucosa that may occur after nasal application (60). Second, the pH should be capable of preserving the stability of the drug within the formulation. Olanzapine is a weak basic molecule so it is stable in a neutral environment (61). Table 4 shows the measured pH values of the formulated nanoemulsions. All of the results were between 6 and 6.6 (slightly acidic to neutral), which is considered to be physiologically compatible with the nasal mucosa and provide stable circumstances for the drug.

Nanoemulsions have tuneable viscosity. They can be made to have a viscosity ranging from thin to very thick gel-like textures (54). The results of the viscosity of the prepared nanoemulsions are listed in Table 4. The viscosity of D2 and D3 formulations were much higher than the others because they contain the higher Tween 80 content when compared to the others. This higher Tween 80 content came from the higher Smix percentage (35% in D2 and 40% in D3) together with the higher Tween 80 to PEG 400 ratio (2:1 in both of them).

Nanoemulsion stability tests

At the end of the heating/cooling cycles, only formulations D4, D7 and D8 maintained their characteristics. Phase separation occurred in the other formulations so they were excluded from further evaluation.

D4, D7 and D8 formulations withstood the next two tests, that is, the centrifugation and freeze/thawing tests with no creaming or phase separation. They were therefore selected for further testing.

In vitro release study

In vitro release studies were conducted on the formulas that fulfil the nanoemulsion stability test requirements. The cumulative percentage of olanzapine release profiles from D4, D7 and D8 nanoemulsions are presented in Figure 3. All the studied formulations showed good release profiles over five hours. The experiments were continued for 5 hours in order to ensure the achievement of a full release profile even if such time frame might not match the retardation time of the nasal formulations in contact with the nasal mucosa, in reality.

D4 formula exhibited the highest cumulative percent of drug release after 5 hours (about 62.20 ± 4.28%) while D7 and D8 showed a much lower cumulative percent of drug release at the end of the test (about

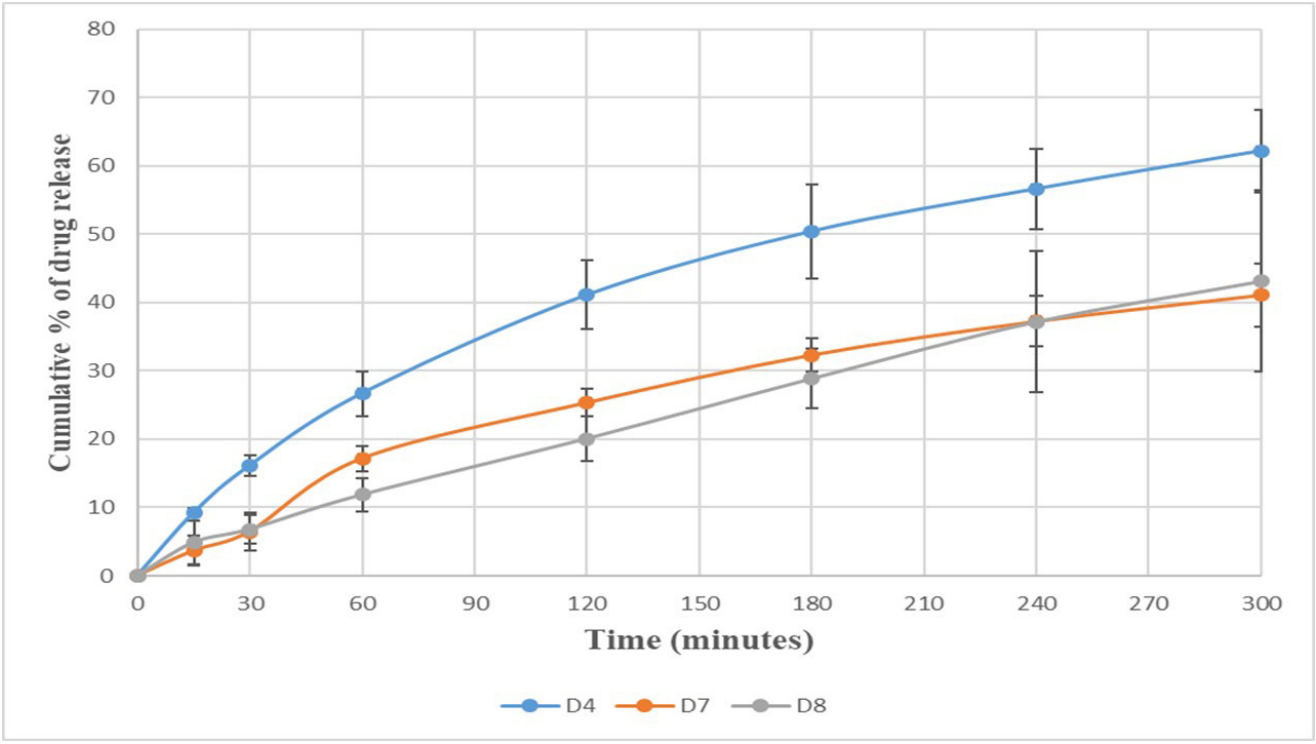

Figure 3 The cumulative % of *in vitro* olanzapine release from D4 (blue), D7 (orange) and D8 (black) formulas *versus* time expressed as mean ± SD (n = 3).

41.1 ± 3.28% and 43.1 ± 9.39, respectively). However, this variation was statistically insignificant (*p*-value ≤ 0.05). The reason for this may be that D4 had a lower percentage oil at 4% compared to 6% in both D7 and D8. This higher oil percentage may contribute to a retardation in the release of the drug out of the oil droplet into the surrounding aqueous phase since with the presence of higher oil (at constant surfactant concentration) will result in the formation of larger droplets (as observed when D4 was compared with D8) leading to a reduction in the surface area with reduced rate of diffusion. However, D7 exhibited a smaller droplet size as compared to D4 but the 5 hours' cumulative percentage of drug release was much lower than D4.

The reason behind this might not fully explained by attribution to particle size. Another possible explanation may be related to the variation in the rheological characteristics among the different formulations. The viscosity of D4 is much lower than that of the two other studied nanoemulsions. This higher viscosity hinders the release of the drug by acting as a barrier that prevent the release media (surrounding the formulation) from penetrating through the nanoemulsion with the resulting decrease in the movement of the drug molecules out of the droplets. This profile is similar to the release profile results reported by earlier studies (62, 63).

Different kinetic models were used to investigate the mechanism of drug release from the formulated nanoemulsions. The *in vitro* release kinetics of D4, D7 and D8 are represented in Table 5. The regression analysis of *in vitro* release kinetic using zero, first and Higuchi models indicated that D4 and D7 follow Higuchi models since it gave the highest R^2 value (0.9923 and 0.9773, respectively). Therefore, their release is diffusion controlled. This behaviour is commonly seen in nano-carrier formulations in many previously published articles (64, 65). This model describes a linear relationship between the cumulative percent of drug release against square root of the time (66). It states that diffusion controls the release rate

Table 5 The kinetic models of olanzapine release from nanoemulsions in PBS solution pH 6.4 (R2 = correlation coefficient)

FORMULATION	R^2		
	Zero-order	First-order	Higuchi
D4	0.93	0.9785	0.9923
D7	0.9427	0.9685	0.9773
D8	0.9933	0.9985	0.9618

of the drug encapsulated within a uniform polymeric matrix if the loading of the drug exceeds its solubility within the matrix according to Fick's law of diffusion (64, 66).

On the other hand, the release profile of D8 formulation was best fitted to the first order model suggesting that the release is directly proportional to concentration (66). The reason behind this finding may be related to the much lower entrapment efficiency demonstrated by D8 compared to those of D4 and D7 (as indicated in Table 3). This lower EE% of D8 causes its release to disobey Fick's law of diffusion (the drug loading lower than the drug solubility) and become concentration dependant.

Evaluation of nanoemulsion based nasal *in situ* gels

The optimized formulation was D4. It exhibited a good droplet size of 110.58±3.07, PDI of 0.202±0.009, a zeta potential of -5.64±0.64 and EE% of 94.74±5.07%. Also, it possessed the least viscosity (7.68±0.06 cP) among all the prepared preparations. During release assessment, D4 demonstrated a very good release with the highest 5 hours' cumulative release %. Accordingly, D4 was selected for incorporation into a gel base formulation.

Sol-gel transition temperature measurement

D4a underwent gelation at 31.1 ± 0.5°C while D4b had a sol-gel temperature of 28.8 ± 1°C. Both of these gelling temperatures are acceptable for nasal *in situ* gels since the internal temperature of the nasal cavity is about 34°C (67).

Ex vivo permeation test

The time frame of the experiment was determined to be 300 minutes since one of the tested formulations contained a muco-adhesive polymer and it was crucial to examine the effect of such polymer on the permeation of the drug over longer time than expected in reality to get a full permeation profile. The cumulative quantities of olanzapine that permeated through the sheep nasal mucosa are represented in Figure 4.

During the first 30 minutes of the test, the rate of diffusion of the drug from D4b was much faster than from D4a. After that, the rate of diffusion decreased for both of them. At the end of the experiment, a cumulative permeation of 479.71 μg/cm^2 and 210.46 μg/cm^2 were obtained for D4b and D4a, respectively.

The flux (J) of D4b and D4a at the steady-state were 0.192 ± 0.071 μg/cm^2.hr and 0.093 ± 0.045 μg/cm^2.hr, respectively, while the permeability co-efficient were 0.03 ± 0.011 cm/hr and 0.014 ± 0.007 cm/hr for D4b and D4a, respectively. This experiment indicates the impact of HPMC addition on the permeability of the drug. There was an approximately one-fold increase in the flux of olanzapine from D4b when compared to its flux from D4a. These observations were expected and they were attributed to the presence of HPMC, which imparts a mucoadhesive characteristic to the formulation with the resulting more intimate contact between the gelled formulation and the diffusing mucosa leading to increased permeation (68).

In vivo pharmacodynamic study (Paw test)

The majority of animals that received olanzapine

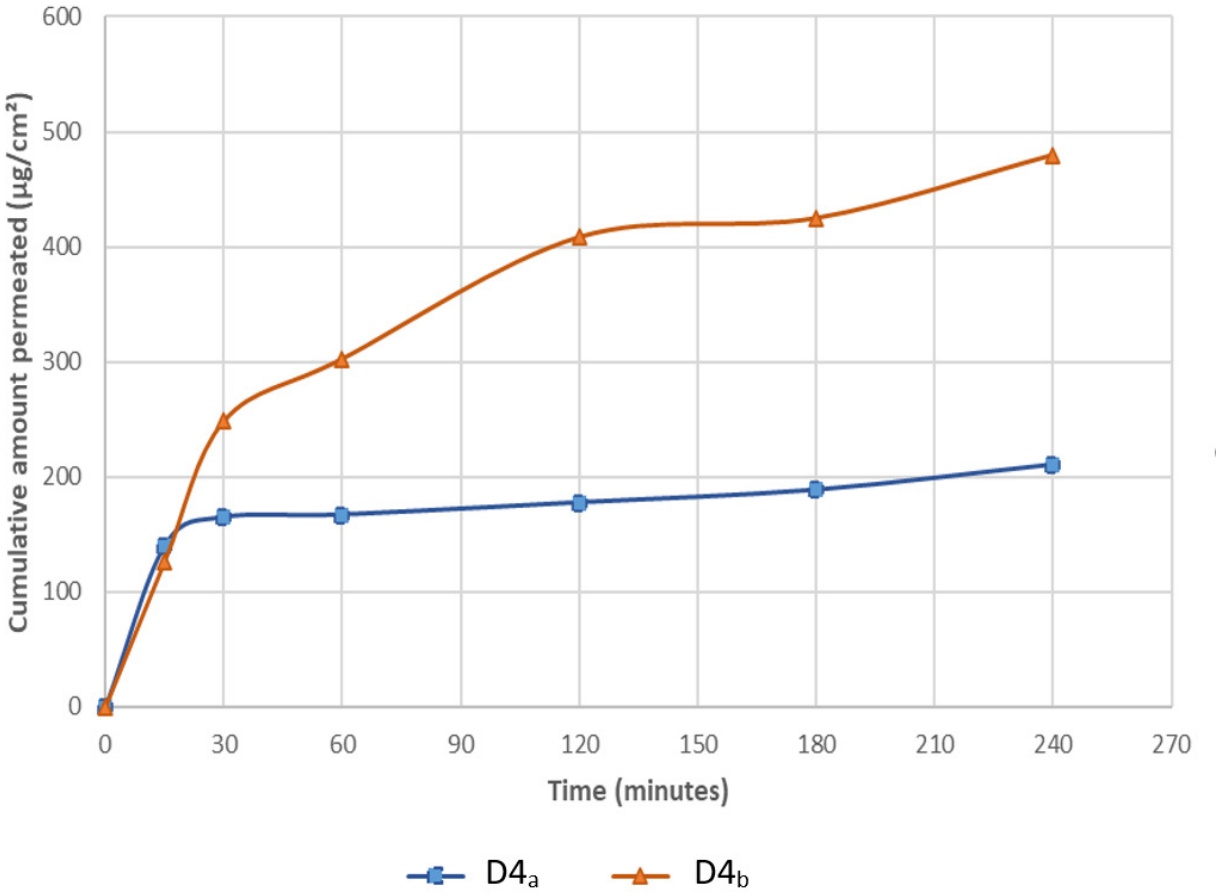

Figure 4 The *ex vivo* permeation of olanzapine from D4a (blue line) and D4b (orange line) across sheep nasal mucosa as a function of time using Franz diffusion cell.

intranasal formulations demonstrated apparent prolongation in the HRT with little effect on the FRT when compared with the negative control group (normal saline intranasally).

On the other hand, the positive control group (haloperidol intraperitoneally) exhibited statistically significant (p-value ≤ 0.05) extension in both FRT and HRT when compared to the negative control group. These observations are in accordance with the literatures published by Ellenbroek et. al, Gupta *et. al.,* and Kumar et. al. which indicated that the second generation (atypical) antipsychotic medications, like olanzapine, influence HRT without causing any modification to the FRT while the first generation (typical) antipsychotics like haloperidol can alter both FRT and HRT (37, 54, 69). These findings (with respect to olanzapine formulations) are favorable since the HRT reflects the antipsychotic efficacy of the medication while FRT correlates with the extrapyramidal side effects (69,70). The results for the pharmacodynamic studies are expressed in Figure 5.

The order for increasing HRT values among the different olanzapine formulations was Osol < OnanoE <D4a<D4b. When comparing the HRT values of the Osol group to the negative control group, there was a statistically significant (p-value ≤ 0.05) increase in the HRT values which provide proof for the good nasal absorption of olanzapine following nasal administration.

The evidence for the enhanced nose to brain delivery of olanzapine (from the particulate system) through the nasal olfactory epithelia following intranasal delivery is provided by the fact that the HRT values are

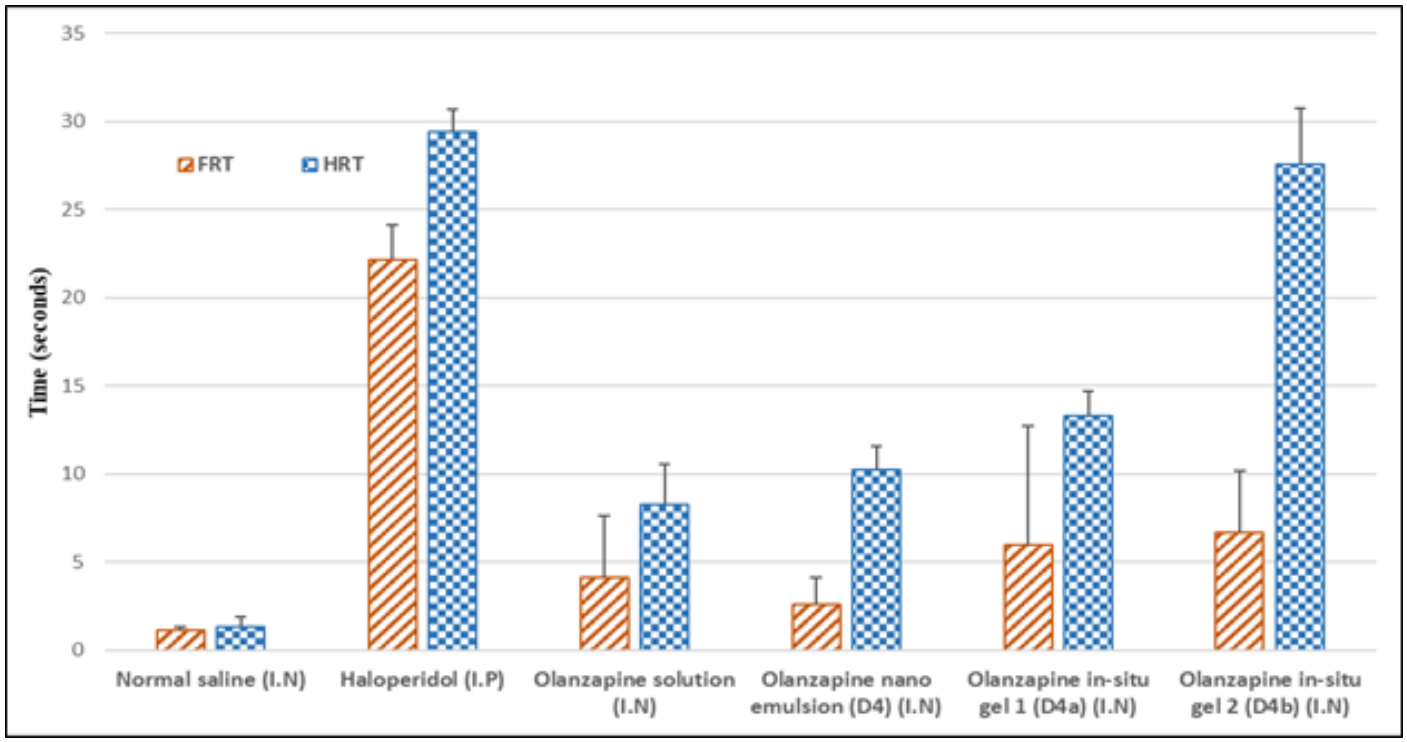

Figure 5 The results of the pharmacodynamic studies (Paw test) of olanzapine preparations when administered intranasally (I.N) in Swiss Albino rats compared to the intraperitoneal administration of haloperidol. FRT (forelimb retraction time) correlates with the extrapyramidal side effects while HRT (hindlimb retraction time) expresses the antipsychotic efficacy. All the results are expressed as mean ± SD (n = 5).

favourable for OnanoE when compared to the Osol even when the difference was statistically insignificant (p-value ≤ 0.05). These results are in agreement with those obtained by previous researchers (37).

D4a also demonstrated statistically significant (p-value ≤ 0.05) higher HRT values when compared to Osol and OnanoE. D4a formulation differs from OnanoE in that it contains Poloxamer 407 (as an *in situ* gelling agent). The *in situ* gelation caused by Poloxamer 407 formed a thick viscous gel that would inhibit the mucociliary clearance and prolong the mucosal residence period.

On the other hand, D4b showed a statistically significant enhancement or extension (p-value ≤ 0.05) in HRT when compared to all other treatments. D4b contains HPMC along with Poloxamer 407. D4b showed the most pronounced effect on the HRT. The possible explanation for that might be first, due to the *in situ* viscous gel near the olfactory mucosa prolong the mucosal contact. Second, the presence of HPMC promotes the mucoadhesion of the formed gel with the absorbing epithelia leading to an increase in the transport of the drug from the formulation to the mucosa with subsequently enhanced absorption and direct delivery to the brain.

Stability testing of the nasal in situ gels

After the storage period, the samples showed no notable change in physical appearance (no phase separation or creaming). Also, there was no statistically significant (p-value ≤ 0.05) change in the measured pH, conductivity and sol-gel transition temperature which indicates good stability of the prepared *in situ* gels. The exception for this is the conductivity measurement for D4b which demonstrated a statistically significant drop in conductivity (p-value ≤ 0.05) following 12 weeks of storage. However, the measured conductivity was still within the acceptable range for oil in water

Table 6 The results of the stability study of $D4_a$ and $D4_b$. The results are expressed as mean ± SD (n = 3)

FORMULATION	pH		CONDUCTIVITY		SOL-GEL TEMP.	
	0 WEEK	12 WEEKS	0 WEEK	12 WEEKS	0 WEEK	12 WEEKS
$D4_A$	6.1 ± 0	6.3 ± 0	120 ± 2.5	115 ± 0.5	31.1 ± 0.5	31.0 ± 0.5
$D4_B$	6.1 ± 0	6.3 ± 0	122 ± 3.0	111 ± 1.0	28.8 ± 1	29.0 ± 0.5

nanoemulsions. The results of the stability study are included in Table 6.

CONCLUSIONS

This study focused on formulating olanzapine as a nanoemulsion providing very good characteristics. In addition, this nanoemulsion was incorporated with a thermo-sensitive polymer (poloxamer 407) to form a nanoemulsion-based nasal *in situ* gel. The addition of HPMC to this *in situ* gel resulted in a nasal drug delivery system that produced a clear superior pharmacodynamics effect when applied *in-vivo* to animals. This nanoemulsion-based nasal *in situ* gel of olanzapine is a promising drug delivery system that might replace other traditional dosage forms such as tablets for treatment of central nervous system diseases.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

The authors contributed equally to the research.

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