Identifying lipidic emulsomes for improved oxcarbazepine brain targeting: In vitro and rat in vivo studies

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Lipid-based nanovectors offer effective carriers for brain delivery by improving drug potency and reducing off-target effects. Emulsomes are nano-triglyceride (TG) carriers formed of lipid cores supported by at least one phospholipid (PC) sheath. Due to their surface active properties, PC forms bilayers at the aqueous interface, thereby enabling encapsulated drug to benefit from better bioavailability and stability. Emulsomes of oxcarbazepine (OX) were prepared, aimed to offer nanocarriers for nasal delivery for brain targeting. Different TG cores (Compertol®, tripalmitin, tristearin and triolein) and soya phosphatidylcholine in different amounts and ratios were used for emulsomal preparation. Particles were modulated to generate nanocarriers with suitable size, charge, encapsulation efficiency and prolonged release. Cytotoxicity and pharmacokinetic studies were also implemented. Nano-spherical OX-emulsomes with maximal encapsulation of 96.75% were generated. Stability studies showed changes within 30.6% and 11.2% in the size and EE% after 3 months. MTT assay proved a decrease in drug toxicity by its encapsulation in emulsomes. Incorporation of OX into emulsomes resulted in stable nanoformulations. Tailoring emulsomes properties by modulating the surface charge and particle size produced a stable system for the lipophilic drug with a prolonged release profile and mean residence time and proved direct nose-to-brain transport in rats.

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1. Introduction

Successful design of nano-vesicular carriers for BBB targeting requires good comprehension of the physicochemical properties and mechanisms involved in the nanocarrier cellular uptake and intracellular disposition. The BBB is a dynamic membrane that restricts the entry of large, hydrophilic, or charged molecules with its hydrophobic part (Khalil et al., 2006). Numerous strategies have been attempted to circumvent the BBB to efficiently deliver drugs into the brain (Illum, 2000). The bypass of the BBB via olfactory and trigeminal-associated extracellular pathways makes the nasal route a path for rapid delivery to the brain, eliciting an early brain pharmacological response. Recent human trials proved the transport of three neurotransmitters to cerebrospinal fluid (CSF) via this route (Illum, 2002). This made scientists keep focus on nasal delivery, added to its main advantage in being noninvasive, in spite of its known limitations like common cold. Not to forget the enhancement of drug bioavailability by avoiding first-pass metabolism, delivery of carrier payload with the desired concentration at the desired site of action and a reduced side effect profile at non-target sites (Patel et al., 2009).

As a general consensus, lipid-based nanovectors hold great potential for delivery to brain when given intranasally. These systems are efficiently taken up by nasal mucosa thereby increasing their bioavailability, specificity, duration of therapeutic action, as well as, offering higher drug loading and protection against enzymatic and chemical degradation (Shah et al., 2015). Many lipid carriers have been investigated for brain targeting, viz., liposomes, solid lipid nanoparticles and submicron lipid emulsions. However, preparation technique shortcomings such as high pressure induced drug degradation, lipid crystallization, gelation and co-existence of several colloidal species are induced (Elgart et al., 2012). Despite initial claims regarding their potential for controlled delivery of hydrophobic drugs, lipid nanoparticles have shown limited controlled release capacity because in most cases the drug and lipids solidify in phase-separated crystals that precipitate either in the core or on the surface of the nanoparticles (Garcia-Fuentes et al., 2005). Consequently, these structures might exhibit slow release or a pronounced burst release.
Emulsomes are nanoemulsions of particles comprising lipid cores stabilized by at least one phospholipid envelope which surrounds the lipid core at the aqueous interface, forming bilayers, thereby conferring stability to the emulsion (Pal et al., 2012). Hence they are considered as nanolipidal carriers stabilized with high percentage of soya lecithin multilayers (Paliwal et al., 2009), developed to synergize the characteristics of both liposomes and emulsomes. A key feature of these particles is that the lipidic core is in a solid or liquid crystalline phase, rather than oil in a fluid phase (Amselem and Friedman, 1997). By this apolar core, emulsomes are differentiated from emulsions by the encapsulation of higher amounts of lipophilic drugs (Uciski et al., 2015) with a prolonged release time (Uciski et al., 2013b). Besides, by their phospholipid surface arranged as lipid bilayers with hydrophilic heads facing outwards and hydrophobic tails lined up against one another, they increase the solubility, as well as, the bioavailability of poorly soluble drugs, resembling liposomes (Chandrika and Babul, 2014). This bilayer with its high phospholipid content covering the lipid core, was also reported to confer high stability to the particles. Additionally, due to their small size, emulsomes have site specificity and thus, increased drug levels at target tissues (Aswathy et al., 2013). A patented form of Co-enzyme Q10 (Ultramsome-CoQ10) showed ten times greater solubility than generic Co-enzyme Q10, with a 100% dissolution capability, proven efficacy and safety in a series of clinical studies and high bioavailability (Berman et al., 2004).

Oxcarbazepine (OX) is a 10,11-di hydro-10-oxo-5H-dibenzo zepin-5-carboxamide, assigned for the treatment of mono- and adjunctive therapy in partial and generalized tonic-clonic seizures in epileptic adults and children (Wellington and Goa, 2001). The recommended daily starting adult dosage is 600 mg/day, which is titrated according to clinical benefit up to 2400 mg/day (Barcs et al., 2000). OX undergoes un-inducible reductive metabolism by aldoketo reductase, forming a monohydroxy derivative that exhibits comparable antiepileptic efficacy (Pucci et al., 2003). Conventional OX administration via the oral route exhibits high systemic distribution to non-targeted tissues, which potentiates the occurrence of undesirable peripheral pathological conditions (e.g. hematologic, hepatic and renal dysfunctions) (Toledano and Gil-Nagel, 2008), besides, drug–drug interactions. Also induction of P450C1 and P450C24, responsible for the metabolism of 25-hydroxyvitamin D, has been reported which predisposes patients to bone loss over time with high OX doses (Mazza et al., 2007). Moreover, marked toxicity has been reported in different types of cells upon treatment with OX (Pavone and Cardile, 2003; Aktas et al., 2009), as well as, the binding affinity of OX to mice DNA in vitro (Myllynen et al., 1998).

Internalization of emulsomes is not yet well studied in the literature, but its cellular uptake is expected to be similar to liposomes, both having identical surfaces (Uciski et al., 2015). To the best of our knowledge, emulsomes have not been fully explored in terms of variations in structure and physicochemical properties for optimum performance; they also have not been tested for intranasal brain targeting. Only minimal work has been published for their use as carriers for certain drugs, viz, methotrexate via the oral route (Paliwal et al., 2009), intracardiac amphotericin B (Pal et al., 2012), curcumin (Uciski et al., 2013a, 2013b), capsain for topical delivery (Gupta et al., 2014) and candesartan cilexetil (Chandrika and Babul, 2014). Hence, with the goal of developing a stable OX encapsulated lipidic nanovector aiming at direct nose-to-brain targeting strategy to improve epileptic therapy, the possibility of using less daily drug amounts can be a platform for circumventing various side and toxic cellular effects. Hence, emulsomes with different triglyceride cores, in different amounts and ratios in combination with soya lecithin, in the presence of additives that adjust nanocarrier charge and size to best suit the target were tried. The safety of the particles was also assessed on the nasal epithelium and the pharmacokinetic performance was evaluated in comparison to the market product, as well as, the drug solution.

2. Experimental

2.1. Materials

Oxcarbazepine (OX) was a gift from Novartis Pharmaceutical Co. (Cairo, Egypt). Compritol® (C) was a free sample from Gattefosse ( Nanterre, France). Triolein (TO) (Captex GTO) was generously supplied by Abitec Corp. (Ohio, USA), Soya phosphatidylcholine (PC), tristerin (TS), tripalmitin (TP), chitosan (Mwt 5000) (Ch) and Triton X were purchased from Sigma Chemical Co. (St. Louis, USA). Tween 80 (Tw), chloroform and methanol were purchased from Adwic, El Nasr Pharmaceutical Co. (Cairo, Egypt). Cellophane membrane (molecular weight cut off 12,000–14,000) was purchased from Spectrum Laboratories Inc. (California, USA).

2.2. Fabrication of emulsomes

Emulsomes were prepared using a modified thin film method previously described by Paliwal et al. (2009). A lipid mixture of PC and different TGs (in several ratios) were used for the preparation of emulsomes, namely, C, TS, TP and TO. The compositions of these formulations are shown in Table S1. After introducing in a clean, dry round bottom flask, the chloroform used to dissolve 10 mg OX mixed with the lipid mixture was removed under reduced pressure by rotary evaporation (Model RV05, Janke and Kunkel, IKA Laboratories, Staufen, Germany). The deposited film was hydrated with phosphate buffer (pH 6.8) by agitation for 1 h at room temperature. The resulting particles were sonicated for 1 min (Bath sonicator model 27ST, Crest Ultrasonics Carp, Trenton, USA) followed by extrusion through 450 nm cellulose nitrate membranes then stored at 4 °C.

Particle coating was done by Tween 80 and chitosan (Mwt 5000) in respective concentrations, (1, 2.5 and 5%) and (0.25, 0.5, 1 and 2% dissolved in HCl at pH 4.5). After separation of the free drug, the emulsomal pellets were incubated for 2 h in the coat solution at ambient temperature and under constant stirring, prior to re-separation of excess coats by centrifugation, and then kept at 4 °C.

2.3. Evaluation of emulsomes

2.3.1. Entrapment efficiency (EE%)

The non-entrapped drug of cooled samples was separated from emulsomes by centrifugation (Hermle cooling centrifuge, Labor technik GmbH, Wertheim, Germany) at 15,000 rpm at 4 °C for 1 h (Chandrika and Babul, 2014). The EE% of oxcarbazepine was determined by disrupting emulsomes using 1% Triton X and analyzing the drug content spectrophotometrically at λmax 305 nm (Enésas et al., 2010) (UV. visible spectrophotometer, model UV- 1601 PC, Shimadzu, Kyoto, Japan). EE% was calculated by the following equation:

\[
EE\% = \frac{\text{Entrapped drug}}{\text{Total drug}} \times 100
\]

(1)

2.3.2. Particle characterization

The average particle size and size distribution of the freshly prepared emulsomes expressed as the polydispersity index (PDI) were estimated by dynamic light scattering technique (DLS), while the zeta potential, ζ, was determined by electrophoretic light scattering by a Zetasizer Nano ZS (Malvern Zetasizer, Malvern
Instruments Ltd., Malvern, UK) equipped with 4 mW 633 nm He-Ne lasers. Samples were properly diluted with deionized water to avoid multi-scattering phenomena and equilibrated at 25 °C. Analyses were performed at an angle of 173° (Seju et al., 2011).

2.3.3. Transmission electron microscopy (TEM)

A sample of the prepared emulsomal particles were imaged by depositing a 50 µL drop of the vesicular dispersion on a carbon coated grid. The droplet was left for 2 min, to allow its adsorption on the carbon film, then excess liquid was drawn off with hardened, fiber-free filter paper. Emulsomes were subsequently negatively stained with a drop of 1% phosphotungstic acid. The excess stain was removed by filter paper and the grid was washed with distilled water then air dried. The sample was examined by TEM (Model JEM-1230, JOEL, Tokyo, Japan) at 200 kV with a magnification of × 25000 (El-Zaafarany et al., 2010).

2.3.4. Drug release

The permeation of OX-bearing emulsomes through cellophane membrane was conducted in Franz-type diffusion cells (VarioMag Telesystem, H+P Laborotechnik, Germany). The donor compartment contained an amount of emulsomes equivalent to 3 mg drug. The receptor compartment, maintained at 37 ± 0.2 °C by a circulating water jacket, contained 7.5 mL phosphate buffer (pH 6.8) and was constantly stirred with a small magnetic bar at 50 rpm (Florence et al., 2011). This low stirring rate was adopted to mimic the low mixing conditions found in the nose (Amidi et al., 2006). Via the sampling port of the receptor compartment, samples of 500 µL were withdrawn at predetermined time intervals of 0.5, 1, 2, 4, 6, 8 and 24 h, then immediately replaced with equal volumes of fresh receptor solution. All samples were analyzed for OX content spectrophotometrically, λmax 305 nm.

2.4. Stability study

Selected emulsomal formulations were subjected to stability study by storage in glass vials at 4 °C for 3 months. Samples from each formulation were withdrawn at definite time intervals (30, 60 and 90 days) and the tested criteria were vesicle size, zeta potential and drug leakage.

Drug leakage was quantified by the drug retained percent (DR%) in which the drug entrapped in the emulsomes, at day zero, represented 100% and those obtained at different times (t) were referenced to it. This was calculated according to the following equation:

$$\text{DR}\% = \frac{\text{Drug entrapped at day } t}{\text{Drug entrapped at day } 0} \times 100$$

2.5. MTT assay

The cytotoxicity of prepared emulsomes were conducted on Calu-3 cells using MTT assay (Elmawal et al., 2014). Briefly, Calu-3 nasal cells were grown on minimum essential medium (MEM), supplemented with: 10% heat-inactivated fetal calf serum, 50 µg/mL gentamicin, 1% l-glutamine and HEPES buffer. The cells were maintained at 37 °C in an atmosphere humidified with 5% v/v CO2 (Humid CO2 incubator, Shel Lab model 2406, USA). Cell seeding was conducted in a 96-well plate, at a cell concentration of 1 x 10⁴ cells per well in 100 µL of the growth medium. Serial dilutions of the tested formulation, its blank, and OX powder were prepared in 100 µL growth medium then added to the cells, followed by incubation for 24 h. 20 µL of MTT solution (5 mg/mL in phosphate buffer saline with pH 7.4) were added to the cell monolayers, and subsequently incubated at 37 °C for another 4 h (Zheng et al., 2012). The medium was then removed and the formed formazan crystals were solubilized by 100 µL dimethyl sulfoxide. The absorbance was read at 570 nm on a microplate reader (Tecan Sunrise® Mannedorf, Switzerland). The percentage of cell viability was calculated by the following equation:

$$\text{Viability} = \frac{A_{\text{test}}}{A_{\text{control}}} \times 100$$

Where Atest is the absorbance obtained for the test formulation and Acontrol is the absorbance obtained for the untreated control cells (incubated with medium only). This latter reading was assumed to correspond to a cell viability of 100%.

2.6. In vivo studies

2.6.1. Pharmacokinetic study

2.6.1.1. Administration to rats. The conducted animal study complies with the National Institutes of Health guidelines for the care and use of Laboratory animals, and the adopted protocol was ethically approved by the “Experiments and Advanced Pharmaceutical Research Unit” of the Faculty of Pharmacy, Ain Shams University, Cairo, Egypt. One hundred and thirty two male Wistar albino rats (200–250 g) were divided into 3 groups, each comprising 44 rats. Group 1 was intravenously (IV) injected with OX solution in phosphate buffer (pH 6.8), Group 2 received the selected IN emulsomal formulation and Group 3 received a market product peroral (Hellewell, 2002). In case of IN and IV administration, OX was administered to the rats in a dose of 0.32 mg/kg, which was in accordance to the dose used by Serralheiro et al. with carbamazepine (Serralheiro et al., 2014). Oral administration of the market product was via intragastric tubing of the suspension in a dose of 30 mg/kg (Matar et al., 1999).

For IV administration, OX solution was injected, as a bolus, into the peripheral rat tail vein. IN instillation was conducted on conscious rats fixed in a prostrate position, and the formulation was administered as a single shot of 20 µL into each nostril using soft polyethylene tubing with an internal diameter of 0.10 mm connected to a microinjector at the delivery site (Gabal et al., 2014). Blood samples were collected from rat eyes at different preset time intervals, viz., 5, 10, 20, 30, 45, 60, 120, 240, 360, 480 and 1440 min following drug administration and inserted in heparinized tubes for centrifugation at 4000 rpm for 10 min in order to separate the plasma. This was followed by sacrifice of the rats for brain isolation, where brain tissues were removed from the cut open skulls, then homogenized (T-25 ultra-turrax digital high speed homogenizer, IKA Werke GmbH & Co. KG, Staufen, Germany) with three-fold volumes saline at 24,000 rpm for 1 min. Separated plasma and homogenized brain tubes were stored at −80 °C until assay.

2.6.1.2. Assay of OX content in plasma and brain. Applied Biosystems/MDS Sciex liquid chromatography tandem mass spectrometry (LC–MS/MS) was used to analyze OX concentrations in the plasma and homogenized brains. Plasma and brain samples were thawed at room temperature, then 0.5 mL were added to 50 µL internal standard solution (toresemide 1500 ng/mL) and 5 mL ethyl acetate. Samples were subsequently vortexed for 1 min, then centrifuged for 10 min at 4000 rpm at 4 °C (Kurti et al., 2013). The organic layers were decanted into Wassermann tubes, then evaporated to dryness using a vacuum concentrator. Reconstitution of dry residues were carried out by the addition of 250 µL mobile phase (80% acetonitrile + 20% water + 0.1% formic acid), then tubes were vortex-mixed for 1 min and finally ready for analysis.
An aliquot of the samples was injected into an LC-MS/MS system (Shimazu Prominence, Kyoto, Japan) equipped with a degasser (DGU-20A3) using Agilent C18 column (50 mm x 4.6 mm x 3.5 μm) (Zorbax Eclipse, Agilent Technologies, California, USA). The isocratic mobile phase was injected into the electrospray ionization chamber of mass spectrometer at a flow rate of 1.0 mL/min. Quantitation of both OX and toremeside was carried out by MS/MS detection in positive ion mode using a MDS Sciex (Foster City, California, USA) API-3200 mass spectrometer fitted with a Turbo Ionspray TM interface, at 450 °C. Ion spray voltage was set at 5500 V with the following common parameters of collision gas, curtain gas, nebulizer gas and auxiliary gas set at 5, 15, 20 and 40 psi, respectively. Compound parameters, viz., entrance potential, collision energy, declustering potential and collision exit potential were set for ideal separation of OX and toremeside. Multiple reactions monitoring (MRM) mode was used for quantification of the ions by monitoring the transitions of m/z 253.1–236 for OX and m/z 348.99–263.9 for toremeside, with Q1 and Q3 quadrupoles set to unit resolution. The constructed calibration curve was linear over a concentration range of 1–3000 ng/mL. All the analytical data were computed by Analyst software (version 1.5).

2.6.1.3. Pharmacokinetic analysis. For assessment of the pharmacokinetic parameters, mean OX concentrations in plasma and brain samples were plotted versus time. Peak plasma and brain concentrations (Cmax), in addition to, the time to reach these peaks (tmax) were determined. Area under OX concentration-time curve (AUC0–1440 min) was calculated by the trapezoidal method (without extrapolation to infinity) using PKSolver add-in software program. The time to reach half the maximum plasma concentration (t1/2), elimination rate constant (Kα) and the mean residence time (MRT) were also calculated. The absolute bioavailability of the IN formulation (AUC0–1440 min)IN and oral market product (AUC0–1440 min)oral compared to IV solution (AUC0–1440 min)IV were calculated using the following equations:

\[
\text{Absolute bioavailability}_{\text{IN}} = \frac{\text{AUC}_{0–1440 \text{ min}}^{\text{IN}}}{\text{AUC}_{0–1440 \text{ min}}^{\text{IV}}} \times 100
\]

\[
\text{Absolute bioavailability}_{\text{oral}} = \frac{\text{AUC}_{0–1440 \text{ min}}^{\text{oral}}}{\text{AUC}_{0–1440 \text{ min}}^{\text{IV}}} \times 100
\]

2.6.14. Evaluation of brain targeting efficiency. To evaluate the brain-targeting efficiency of emulsomes in comparison to the market product, two indexes were adopted:

i) Drug targeting efficiency (DTE%) which represents the time average partitioning ratio (Amidi et al., 2006)

\[
\text{DTE }\% (\text{IN}) = \frac{\text{AUC}_{\text{brain}}^{\text{IN}} / \text{AUC}_{\text{blood}}^{\text{IN}}}{\text{AUC}_{\text{brain}}^{\text{IV}} / \text{AUC}_{\text{blood}}^{\text{IV}}} \times 100
\]

\[
\text{DTE }\% (\text{oral}) = \frac{\text{AUC}_{\text{brain}}^{\text{oral}} / \text{AUC}_{\text{blood}}^{\text{oral}}}{\text{AUC}_{\text{brain}}^{\text{IV}} / \text{AUC}_{\text{blood}}^{\text{IV}}} \times 100
\]

ii) Direct nose-to-brain transport percentage (DTP%) (Vyas et al., 2006)

\[
\text{DTP }\% = \frac{(B_{\text{IN}} - B_{\text{x}})}{B_{\text{IN}}} \times 100
\]

\[
B_{\text{x}} = \frac{B_{\text{IV}}}{P_{\text{IN}}}
\]

Where, \( B_{\text{IN}} \) is the AUC0–1440 min(brain), following IN administration. \( B_{\text{x}} \) is the brain AUC fraction contributed by systemic circulation through the BBB following IN administration, \( B_{\text{IV}} \) is the AUC0–1440 min(brain) following IV administration, \( P_{\text{IN}} \) is AUC0–1440 min(blood) following IV administration and \( P_{\text{IN}} \) is AUC0–1440 min(blood) following IN instillation.

2.6.2. Histopathological study

Three male Wister albino rats weighing 180–220 g received a single daily intranasal administration of 20 μL emulsomal

![Fig. 1](image-url)
formulation (either plain or drug loaded) in one nostril and normal saline as a control in the other nostril, for 14 consecutive days. Subsequently, the rats were sacrificed and the nasal septums with their epithelial cell membranes were isolated and placed in 10% formaldehyde for 24 h. This was followed with decalcification, washing with tap water and then dehydration by serial dilutions of alcohol (methyl, ethyl and absolute alcohol). Specimens were cleared with xylene, embedded in paraffin then placed in a hot air oven at 56 °C for 24 h (Salama et al., 2012). Subsequently, tissue blocks of paraffin beeswax were divided into sections of 4 μm, by slide microtome. The obtained tissue sections were mounted onto glass slides, deparaffinized and stained with hematoxylin and eosin. Slides of treated and untreated tissues were examined with a light microscope (Olympus optical microscope, Tokyo, Japan).

2.7. Statistical analysis

All data obtained in the in vitro experiments were expressed as mean ± standard deviation (SD) (n = 3), whereas, for the animal study, the results were mean ± standard error (n = 4). Significant differences in mean values were statistically evaluated by Student t-test, paired and unpaired, for dual comparisons and one way analysis of variance (ANOVA) for multiple variables, using a statistical software program (GraphPad Instat, USA). A p-value less than 0.05 was considered to be significant.

3. Results and discussion

3.1. Optimization of emulsomes

As previously defined, emulsomes are nanoparticles formed from lipid cores (generally TG) surrounded by a PC envelope at the aqueous interface, forming bilayers, thereby stabilizing the emulsion. Ox emulsomal vesicles were prepared by thin film hydration method; using various TGs, different PC to TG ratios and total lipid amounts. All the selected lipids were biocompatible and biodegradable. C, TS and TP were selected as examples of high melting point saturated triglycerides with different fatty acid (FA) chain lengths, viz. C22 (mainly glyceryl dibehenate), C18 and C16, respectively. Their respective melting point ranges are 69–74, 54–72.5 and 44.7–67.4 °C. Whereas, TO was an example of a low melting point unsaturated triglyceride with a C18 chain length and –4 °C melting point.

3.1.1. Entrapment efficiency

Being a lipophilic CNS acting drug, Ox was expected to benefit from the emulsomonal formulation to achieve high drug load. In this study it was found that the lipid amounts and types composing the prepared emulsomes affected their EE%, where the calculated values ranged from 8.85 to 96.75% (Supplementary data, Table S1). Fig. 1 shows the maximum drug EE% attained by all TGs at each ratio. For example, C was the best lipid in case of 0.5:1 PC to TG ratio with a recorded EE% of 23.09, TP for the ratios 1:1 and 2:1 with respective values of 88 and 91.04% and TO for 3:1 ratio with a drug EE% of 96.75% (p < 0.05).

C superiority at lower content of PC could be due to its more pronounced hydrophobic property, conferred by its longer chain length (C22), which enhances the intermolecular entrapment of the drug by inter chain intercalation (Aburahma and Badr-Eldin, 2014). Also, its complex nature and hence, less perfect orientation is a contributing factor, being a blend of different esters of behenic acid with glycerol (mono- (12–18 w/w), di- (45–54 w/w) and tri- (28–32% w/w behenate)). This complex nature, at a low PC content, formed an imperfect crystal lattice that provided hollow spaces for Ox accommodation (Rawat et al., 2011). TO, on the other hand, showed the absolute maximum encapsulation of Ox at the ratio 3:1, which appeared to be optimum ratio for its chemical nature (unsaturated TG with C18 carbon chain), since it has been reported that TO considerably decreases the bending rigidity of membranes avoiding drug leakage (Duelund et al., 2013). TS with saturated C18 chain turned out to be the least one favoring maximum Ox loading into emulsomes.

According to the type of TG, a general trend for the effect of increasing PC to TG ratios on the EE% of the drug was noticed. C and TO showed an increase in EE% with increasing ratios, indicating the positive effect of PC content on drug loading abilities. TS showed an optimum ratio of 2:1, to achieve its maximum EE% (80.7), while TP showed an initial rise in EE% between ratios 0.5:1 and 1:1, followed by almost constant values at higher PC to TG ratios. The 0.5:1 ratio exhibited exclusively lower Ox EE% due to insufficiency of PC content in emulsomes.

Finally, the lipid amounts (15–75 mg) also influenced the EE% of Ox, depending on the PC to TG ratio. The increase in lipid amounts generally increased the EE% but with different patterns according to the type of TG and PC to TG ratio. Accordingly C, TP, TS and TO increased EE% up to a certain limit (30 or 45 mg) with 1:1 and 2:1 ratios followed by a decline. This was true in all cases except TS at the 1:1 ratio and TO at the ratio 3:1, where no decrease was noticed with higher lipid amounts which might be due to the effect of higher PC, masking the individual effects in TGs chemical nature.

Being a lipophilic drug, with logP 1.24 (Douroumis and Fahr, 2006), Ox was successfully loaded in emulsomes either in lipidic core or in the phospholipid bilayers with interlamellar spacing (Ucisik et al., 2013a). This tight orientation functions to ensure stabilization of the core conformation, hence a low chance of drug leakage is anticipated. Additionally, the increase in PC content accompanying higher PC to TG ratios could lead to the formation of PC multilayers around the lipid core, hence, allowing the
intercalation of OX into these bilayers (Pal et al., 2012). Counterintuitively, although the long-chain fatty acids attached to the glycerides are expected to increase the accommodation of lipophilic drugs, steric henderence between these chains could have created in some cases voids and imperfections in the lipid matrix (Dudhipala and Veerabrahma, 2014).

3.1.2. Size and charge

Lipid-mediated free diffusion across the BBB must fit the dual criteria of a 400 Da molecular weight threshold, in addition to lipid solubility (Pardridge, 2012). Successful nose to brain delivery is mainly achieved via the olfactory neural pathway which involves axonal transport and bulk flow transport through perineural channels, accordingly, particle size control is crucial.

Table S2 (Supplementary data) shows the sizes and PDI of all the prepared formulations. Emulsomes varied in size from 120.4 nm ± 1.45 (TO17) to 567.8 nm ± 12.4 (TP19) with PDIs ranging from 0.2 to 0.6. Most of the lower PDI values were encountered with TO. The TGs chemical nature obviously affected the particle sizes of emulsomes; C and TO showed generally smaller sizes than TS and TP, probably due to better packing with PC and assembly of the vesicles. Fig. 2 shows that 80% and 95% of emulsomes prepared using C and TO, respectively, had average sizes <300 nm with no formulations >500 nm. Whereas, TS and TP produced 15% and 5% of the prepared emulsomes with average sizes >500 nm and only 60% and 25% with sizes less than 300 nm, respectively.

A marked increase in size associated the increase in PC to TG ratio at each total lipid amount for C, TS and TP (p < 0.05), as depicted in Fig. 3a (C as a model). This confirms the aforementioned hypothesis that increasing the phospholipid concentration causes the formation of multiple bilayers, increasing size and consequently the EE%. On the contrary, TO vesicles became smaller in size upon increasing the phospholipid content with an associated increase in EE% (Fig. 3b).

Emulsomes exhibited a highly negative surface charge in all formulations, in the range of –28 to –67 mV (Supplementary data, Table S3), which reflects the great ability of emulsomes to completely encapsulate OX with a measured ζ of 21.4 ± 1.25. This positive value is due to the basic character of the drug with a pKa of 10.7. It is currently admitted that zeta potential – 30 mV is required for electrostatic stabilization (Dudhipala and Veerabrahma, 2014). As per the structure of emulsomes, most probably soya PC is the main contributor to this negative charge (Xu et al., 2011). Contradicting results were reported in this area; chylomicron mimicking methotrexate emulsomes with C core prepared by Palival and co workers were negatively charged (Paliwal et al., 2009), whereas, curcumulomes with TP core exhibited positive charges (Ucisik et al., 2013b).

3.1.3. Drug release

In order to evaluate OX release from emulsomes, formulae with sizes equal to or less than 200 nm with not less than 60% EE% were chosen (C13, TO8, TO13, TO17 and TO18).

Generally, as depicted in Fig. 4, a comparatively rapid release of OX during the initial phase (first 2 h) was encountered, followed by a slower sustained release up to 24 h. Drug release was extended in all formulations, with values ranging from 10.79% in case of TO8 to 36.74% and 41.81% for C13 and TO17, respectively. The lipophilic nature of OX would have favoured its residence in the lipidic core of emulsomes. Similarly, Kuo and Chung showed that the release of stavudine, delavirdine, and saquinavir from solid lipid nanoparticles with a core comprising C and TP TGs was based on the relative solubility of the tested drugs in the water-based buffer in contrast to the lipid core (Kuo and Chung, 2011). Moreover, the release patterns of these emulsomes resemble the drug enriched core model surrounded by a solid lipid shell described by Wissing and coauthors (Wissing et al., 2004).

The drug release from the tested formulae can be arranged ascendingly in the following order: TO8 < TO18 < TO13 < TO17 = C13.

The highest amount of OX released over 24 h was evident with C13 and TO17, which showed comparable amounts of drug release after 24 h (p > 0.05). C13 was prepared from C with 2:1 PC to TG ratio and 45 mg total lipid, while TO17 was made of TO with a

![Fig. 3. Effect of PC to TG ratio and lipid amount on the sizes of emulsomes prepared using compritol (a) and triolein (b).](image-url)
3:1 ratio and 30 mg total lipid, indicating that neither the PC:TG ratio, the type of lipid, nor the total lipid amount affected the drug release. In this case OX release was affected by other parameters such as lipid nature, melting point and drug solubility.

3.2. Evaluation of the effect of additives for further optimized brain delivery

The type and magnitude of charges of nanoparticles play key roles in determining their *in vitro* stability and *in vivo* pharmacokinetics and biodistribution. Pillay and Choonara (2015) reported
that highly negative particles could be repulsed at the BBB. Hence, Tween 80 and low molecular weight chitosan were tried to reduce the net charge on emulsomes showing the highest amount of OX released after 24 h and suitable particle size (C13 and TO17).

Chitosan (Ch), being a biodegradable cationic polysaccharide (Sarvaiya and Agrawal, 2015), was chosen as a safe candidate for particle coating. Low molecular weight chitosan was used to avoid particle size increase. Various concentrations of chitosan resulted in non-significant incremental increases in absolute $\zeta$ of C13-Ch and TO17-Ch with maximum variation around 20% in both cases (Fig. 5a). Additionally, sizes of C13-Ch and TO17-Ch markedly increased from 202.2 to 381.6 nm and from 120.6 to 318.2 nm, respectively, which was not a desirable attribute. So it was deduced that the tried chitosan, although being chosen with a low grade viscosity and used in limited concentrations, it increased the particle size significantly without producing the required $\zeta$ decrease.

Coating emulsomes with increasing concentrations of Tween 80 resulted in marked rises ($p < 0.001$) in $\zeta$ from $-45.7$ to $-28.6$ mV (37.4% elevation) and from $-34$ to $-6.72$ mV (80.2% elevation) for C13-Tw and TO17-Tw, respectively (Fig. 5b). It also had the double advantage of reducing their sizes from 202.2 to 187.6 nm for C13-Tw and from 120.6 to 101.5 nm for TO17-Tw ($p < 0.001$). A similar observation was noted for docetaxel loaded poly-$\varepsilon$-caprolactone-Tween 80 copolymer nanoparticles intended for cancer treatment, which was justified by the self-emulsifying function of Tween 80 (Ma et al., 2011). However, this finding is in disagreement with Das and Lin (2005) who showed that double coating of butylcyanoacrylate-dalargin loaded nanoparticles with Tween 80 had no significant effect on the particles size of the nanoparticles.

Myers reported that non-ionic surface active species adsorbed on the surface force the shear plane in the stern double layer further away by shielding it, thus reducing net charge (Myers, 1991). Therefore, higher Tween 80 amounts reinforce the protective effect of the adsorbed polyoxyethylene chains on emulsome surface. Similarly, Sun and coworkers illustrated an approach to produce positive shift in $\zeta$ for enhanced brain targeting of polyactic acid nanoparticles (Sun et al., 2004). Tian et al. (2011) showed that temozolomide loaded polybutylcyanoacrylate nanoparticles coated with Tween 80 had better brain biodistribution than uncoated ones.

**Fig. 6.** Changes of particle size (a), zeta potential (b), and percentage of OX retained (c) in selected emulsomes during a 3-month storage at refrigeration temperature.
The reduction of both the absolute value of $\zeta$ and vesicular size are desirable attributes for effective brain targeting. Besides, the release studies of the coated formulae C13-Tw and TO17-Tw exhibited significantly higher cumulative amounts starting after 4 and 8 h, than their corresponding uncoated ones with 1.25 and 2 folds increase after 24 h, respectively. The lodging of Tween 80 in the phospholipid bilayer might have caused leakage points for the drug to be released. Previous findings revealed drug leakage from Tween coated methotrexate nanoparticles (Trapani et al., 2011). In contrast, other researchers reported only minimal affection of drug release due to Tween 80 coating, which was justified by drug leakage during coating (Tian et al., 2011) or surfactant shielding effect (Kura et al., 2014).

It was previously concluded from the release study that neither the PC toTG ratio, nor the type and amount of lipids, affected the drug release. However in presence of tween 80, it appeared that more leaky emulsomes were obtained with TO based emulsomes than in C ones. The shorter chain lengthed-cores of the former fatty acid helped in a better surfactant assembly than with longer chain fatty acids of C into the bilayers.

### 3.3. Stability study

The ability of emulsomes to retain the incorporated OX and to preserve their particle size, $\zeta$ and% drug retained during refrigerated storage for 3 months was assessed. Tween 80 coated formulae (C13-Tw and TO17-Tw) were subjected to stability studies and compared to their respective uncoated ones (C13 and TO17).

Upon storage, the uncoated formulae showed minute increases in sizes (Fig. 6a) with insignificant alterations in surface charge (Fig. 6b) and DR% at each time interval (Fig. 6c) ($p > 0.05$). Tween 80 coat conferred slight reduction in the surface charge associated with relatively small particle size increase by 30.6%, $\zeta$ decrease by 10 mV, as well as, a reduction in drug EE% by 11.2% upon storage. However, all these alterations were within acceptable limits. Likewise, Uner and co-workers reported small changes in the size and $\zeta$ of Tween 80 stabilized solid lipid nanoparticles, but claimed nonetheless the stability of the particles (Uner et al., 2004). As described earlier, the less ordered structure of the lipid matrix that provides increased imperfections for permittivity of higher drug incorporation, also results in physical stability and little effusion of the drug molecules from particles during storage time (Lopes et al., 2012).

![Fig. 7. TEM micrograph of TO17-Tw emulsomes.](image)

![Fig. 8. Viability of Calu-3 cells by MTT assay after incubation with various concentrations of the test samples at 37 °C.](image)
These results confirm the unique stable structure of emulsomes, regardless the fatty acid core chain lengths, due to solid cores and stabilizing phospholipid coats.

Since both emulsomal formulations were proved stable but TO17-Tw exhibited more complete release, it was selected for further investigation, including morphology, cell toxicity and animal studies.

3.4. Morphology

TEM photomicrograph of TO17-Tw emulsomes depicted the almost spherical shape of scattered morphologically similar vesicles (Fig. 7). The consistent appearance of phospholipid bilayers around solid lipid core is obvious as a brighter periphery which differentiates emulsomes as a carrier from other solid lipid nanoparticles.

3.5. Nasal tolerability studies

3.5.1. Cytotoxicity study

The effect of different concentrations of OX, plain and drug loaded TO17-Tw on the cell viability of Calu-3 cells after a 24 h exposure was evaluated using the MTT assay. As illustrated in Fig. 8, the drug alone showed the highest toxicity to Calu-3 cell line (p < 0.001). This could be due to the positively charged nature of OX. Similarly, Ambrosio et al. reported the neurotoxic effect of OX on hippocampal neurons (Ambrosio et al., 2000). Stress induction by OX on astrocyte culture cells, proving its lack of tolerability by cortical astrocytes was also reported (Pavone and Cardile, 2003).

A higher cell viability (>80%) is clear after drug incorporation in emulsomal formulation, up to 62.5 μg/ml, after which the viability exhibited a normal concentration dependant decrease (Elmowafy et al., 2014). The noxious effect of the positive charges and the control of drug release, thus exposing the cells to lower drug concentrations, might have been the reasons to induce better cell

Fig. 9. Light photomicrograph of rat nasal epithelium treated with saline as a control (a), plain TO17-Tw (b) and OX incorporated TO17-Tw (c). Magnification size of (a) is X 40, while (b) & (c) is X 16. (no: nose, mu: mucosal lining epithelium, v: blood vessels & m: inflammatory cells).
safety. In the next section further in vivo studies in rats were performed for the chosen emulsomal formulation.

3.5.2. Nasal histopathological evaluation

In vivo nasal histopathology studies were conducted to check the possible changes in rat nasal mucosa induced by plain and medicated TO17-Tw emulsomes (Fig. 9). The study revealed no histopathological alteration and normal histological structure of the lining epithelium of the nasal cavity and the underlying cartilaginous tissue in rats treated with plain emulsomes (Fig. 9b). Our results are on similar lines with the reported safety of phospholipids (Chen et al., 2009), TO (Zhang et al., 2011) and Tween 80 (Acharya et al., 2013).

Small alterations in the nasal tissues were observed in the nostril treated with TO17-Tw, where the epithelial lining was intact but with minimal focal inflammatory cells infiltration in the lamina propria and mild congestion in the blood vessels of the mucosal layer (Fig. 9c), as compared to the nostril receiving saline (Fig. 9a). This mild inflammation may be due to the controlled release of the drug throughout the study. This observation of mild cell toxicity induced by the liberated OX is in agreement with Aktaş et al. that proved significant retinal ganglion cell toxicity in rats treated with OX (Aktaş et al., 2009). Conclusively, these findings confirm the cell culture results.

3.6. Pharmacokinetics

Intranasal delivery holds great promise for targeting drugs to CNS due to the high vascularity of the nasal mucosa available for rapid absorption of drugs to systemic circulation, as well as, the direct nose-to-brain delivery via the olfactory region. OX concentrations in plasma and brain after the administration of IV OX solution, IN TO17-Tw emulsomes and oral marketed product at predetermined time intervals are depicted in Fig. 10, and the corresponding pharmacokinetic parameters are measured in Tables 1 and 2.

Fig. 10a reveals that TO17-Tw emulsomes, OX IV solution and marketed product all reached comparable Cmax values (p > 0.05), however, TO17-Tw and IV drug solution only had comparable higher AUC 0-1440min (p < 0.05). This high systemic absorption of TO17-Tw could be clarified by the lipophilic nature of emulsomes that allowed partitioning of the particles into nasal epithelial cell

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**Fig. 10.** Mean OX concentrations in the plasma (a) and brain (b) of rat groups receiving TO17-Tw emulsomes, IV drug solution and oral market product.
membranes into systemic circulation. Furthermore, TO17-Tw emulsomes showed a MRT in plasma 18.8 times that of IV solution and 4.66 times that of the oral formulation. Significantly larger residual amounts of OX after 24 h were quantified in the plasma with emulsomes than the marketed product (p < 0.01), which was anticipated by the proved sustained release of OX from emulsomes. The plasma half-life of TO17-Tw was 18 times that of IV solution and 4.5 times that of marketed product. It has been reported that nanoparticles of size below 200 nm have long blood circulation and thus escape the RES and bypass the liver and spleen filtration (Kaur et al., 2008).

These pharmacokinetic results suggest that encapsulation of OX in emulsomes slowed its elimination, retained a high concentration in the bloodstream for a longer time-period, and increased the circulation time of OX in rats, when compared to the free drug (Zhou and Chen, 2015).

The mean OX concentrations in the brain of rats after administering all tested formulations are depicted in Fig. 10b. Emulsomes showed higher C_{max} nearly 5 times IV solution and market product, and significantly earlier T_{max}. In contrast to plasma data, the emulsomes showed significantly higher brain AUC_{0–1440 min} than IV solution and oral product (p < 0.05). A direct nose-to-brain delivery was hence proved, in addition to a transmembrane uptake across BBB.

The extent of nose-to-brain delivery via the olfactory region has been evaluated by drug targeting efficiency (DTE%) (Abdelbary and Tadros, 2013) and nose-to-brain direct transport (DTP%) (Zhang et al., 2004). Table 2 shows a 3.2-fold higher DTE% of emulsomes in comparison to oral suspension (p < 0.01). DTP% represents the amount of drug (as a percentage) directly transported to the brain via the olfactory pathway by subtracting the amount contributed by systemic circulation (Vyas et al., 2006). The high value in case of TO17-Tw emulsomes further suggests that drug transport to brain via the olfactory route is a main path. It should be pointed out that DTP% of the marketed product showed a negative value due to its small AUC_{0–1440 min} in brain (B_{NN} < B_{C}). Hence, the high DTE% and DTP% for emulsomes indicate their efficiency in targeting drugs to the brain directly through the nose. Direct nose-to-brain drug delivery underlies three mechanisms; two extracellular transport mediated routes which allow rapid entrance of drugs into brain within minutes of IN administration and an intracellular transport-mediated route that accounts for slow drug uptake (Haque et al., 2012).

The exact mechanism of nanoparticle mediated transport of drugs across the BBB has yet to be determined, proposed theories are; higher retention time of nanoparticles in brain capillaries; transcytosis/endocytosis of nanoparticles by brain endothelial cells; and the surfactant/phospholipid fluidizing effect on endothelial cell membrane and thus enhanced drug permeability. Tween 80 was also reported to help in transport through the blood brain barrier by inhibiting P-glycoprotein efflux pump (Kreuter, 2001). Scientific evidence shows that nanoparticles coated with Tween 80 could mimic low-density lipoproteins by covalently coupling with apolipoprotein A-I, B-100 or E in the blood stream, thus interacting with low density lipoprotein receptors, resulting in their uptake by brain capillary endothelial cells (Michaelis et al., 2006). Additionally, Sun et al. proved that coating nanoparticles with Tween 80 was necessary for brain delivery, confirming that nanoparticle-mediated transport of drugs across BBB was most likely by endocytosis (Sun et al., 2004).

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2016.02.038.

**References**


