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# Intranasal Oleic acid-based nanoemulsion of Diazepam: design, formulation and *in-vitro* evaluation

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**ABSTRACT**: An O/W nanoemulsion of Diazepam for intranasal administration was formulated aiming at selecting appropriate surfactant components (surfactants and cosurfactants) and ratios when using oleic acid as an oil phase. Triangular ternary phase diagrams were constructed by specialized software. Tween 80 and cremephore EL were studied as surfactants. Ethanol was studied as co-surfactant. Those were used as emulsifiers with ratio 1:1, 1:2 and 1:3. Fourteen nanoemulsion formulas were formulated by low energy emulsification using aqueous titration method.

The nanoemulsions were characterized for visual transparency, average globule size, polydispersity index, zeta potential, pH, % transmittance and in-vitro drug release.

The formula F3 was chosen as optimum Diazepam nanoemulsion according to results of characterization. Nanoemulsion F3 had a $S_{mix}$ (Tween 80 to Ethanol 1:2): oil: deionized water (50: 10: 40) ratio offered best results with good globule size range (7.5 nm), good PDI (0.22), good percent transmittance (98.5%), pH of 5.7 and highest percent released of Diazepam within the first hour.

**KEYWORDS**: Nanoemulsion; Intranasal; Diazepam; Ternary Phase Diagrams; Intranasal Delivery; In vitro Drug Release

## 1. INTRODUCTION

The nasal route of drug administration has several advantages over oral or intravenous administration. These advantages are not special per se to the nasal route but they can't be achieved by the oral and intravenous route all at the same time, of which are non-invasiveness, self-administration, shorter time to onset of effect, higher bioavailability due to avoidance of hepatic first-pass metabolism and, more importantly, bypassing the blood brain barrier with the potential increase in central nervous system availability of the drug [1].

The types of nanocarriers used in systemic intranasal delivery of drugs include nanomicelles, nanoemulsions, liposomes and nanoparticles (nanospheres and nano-capsules). These systems are generally used to improve the bioavailability of drugs by increasing their diffusion through biological membranes, to protect them against enzyme inactivation and granting blood brain barrier access of non-transportable drugs by masking their physicochemical properties by encapsulation in these systems [2].

Nanoemulsions are characterized as thermodynamic stable isotropic mixtures of oils, surfactants, and/or co-surfactants [3]. These liquid mixtures provide a reduction in volume of dose and can be also designed as a spray formulation [4]. Nanoemulsions are two immiscible liquids dispersed and stabilized by means of adding appropriately mixed ratios of surfactants and co-surfactant, with a preferable droplet size below 100nm [5]. The O/W nanoemulsions are the ones that are commonly used for nose to brain drug administration.

Diazepam is a benzodiazepine that act as positive allosteric modulators of the GABA inhibitory neurotransmitter in the CNS and is a mainstay in managing epileptic emergencies, however; it can't be administered orally during an attack and establishing an IV line for its IV administration during an attack is particularly difficult for responding emergency medical staff and formulating it as a nasal spray can add a considerable advantage in managing such attacks [6].

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## 2. RESULTS & DISCUSSION

### 2.1. Construction of Calibration Curves

Figures 1 & 2 demonstrate the calibration curves of Diazepam obtained in Phosphate buffer of pH 6.4 and in pure ethanol respectively. Diazepam calibration curves showed a linear regression with $R^2$ values of 0.9994 and 0.9995 respectively. These values indicate the linearity correlation between concentration and absorbance and verify that the calibration curves comply with Beer's law within the range of concentrations used.

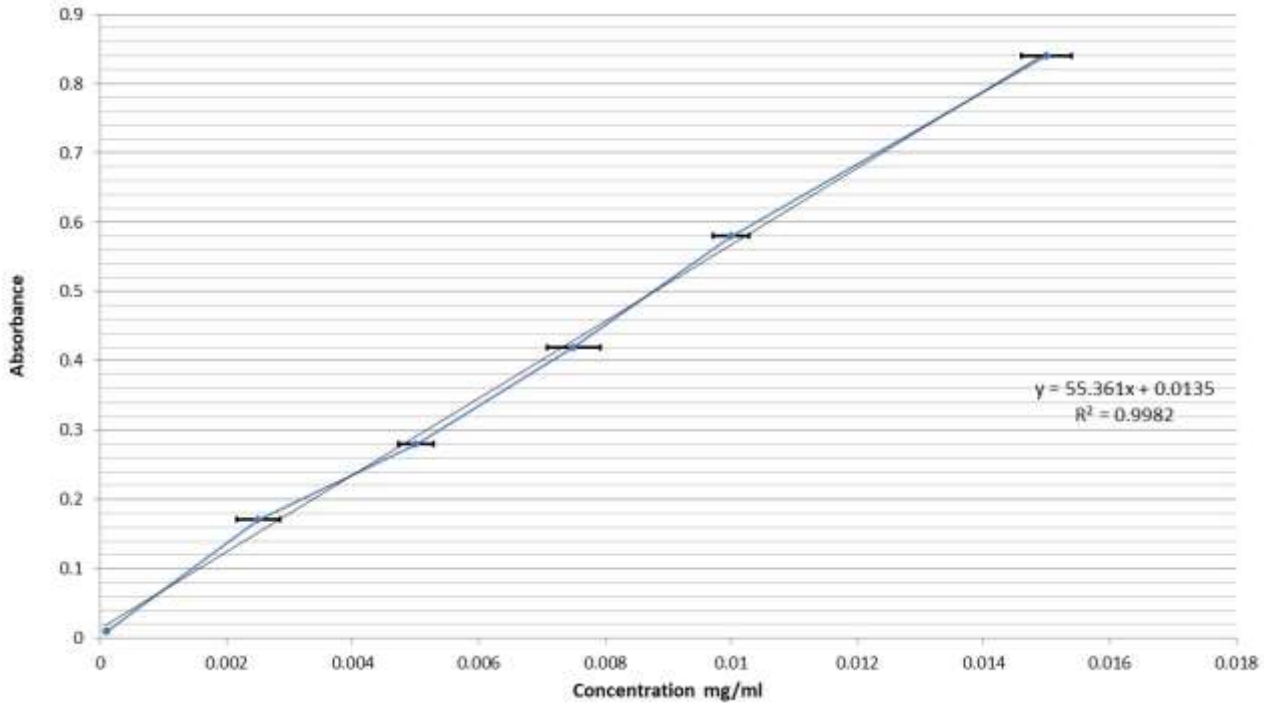


*Figure 1: Calibration curve of diazepam in phosphate buffer 6.4*

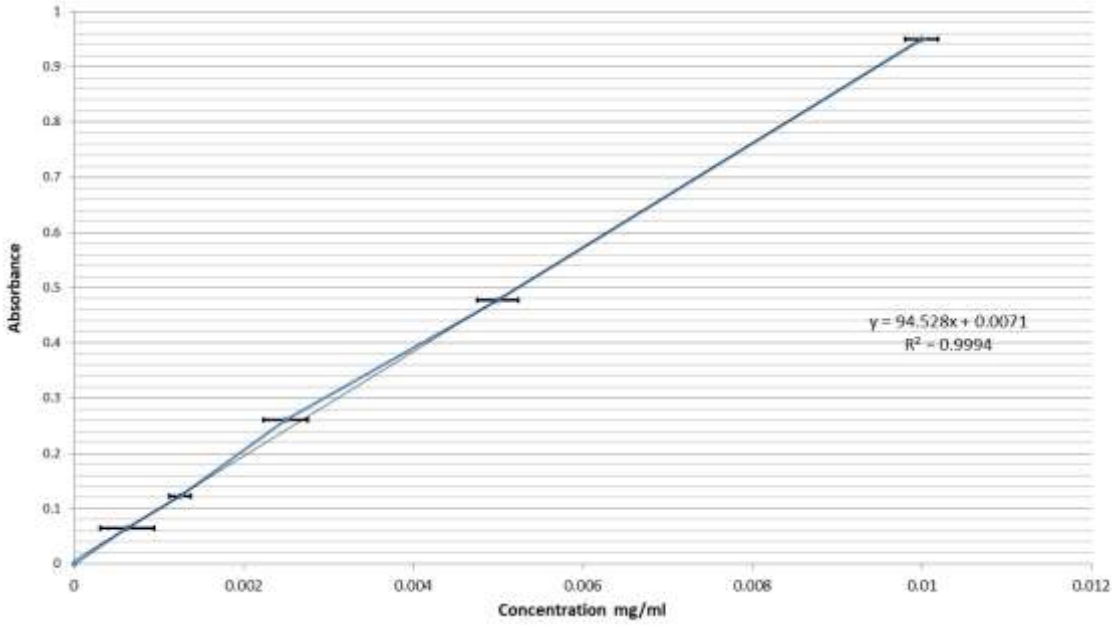

*Figure 2: calibration curve of diazepam in ethanol*

### 2.2. Diazepam Solubility in Different Emulsifiers

The measured solubilities of Diazepam in surfactants and cosurfactants were found as 36mg/ml in Tween 80, 60mg/ml in Cremophor EL and 43mg/ml in ethanol. It's worth mentioning that since the solubility of Diazepam is very low and classified as practically insoluble in water [7], it's up to the non-aqueous components of the emulsion to contain the desired dose.

Diazepam dose in marketed hydroalcoholic preparations is 5 mg/ml with a total volume of 2 ml that is usually injected intravenously or intramuscularly as a single dose. It's essential to achieve the same concentration of the drug in the intranasal emulsion and since the diazepam solubility in the hydrophilic phase of the emulsion is negligible (0.04mg/ml)[8], the maximum volume that can be successfully delivered to humans being around 0.4ml with lower values being preferred and more convenient [9], the total dose amount of diazepam should be dissolved in very limited amount of oil in the formula since higher oil percentages in a O/W emulsion will reduce physical stability of the resultant emulsion and require higher amounts of surfactants and/or cosolvents[10].

### 2.3. Construction of Pseudo-Ternary Phase Diagrams

On the projected and visualized phase diagrams, the light gray patches that are encircled by demarcations provide an approximate indication of the zone where nanoemulsion are possible to be formulated while the rest of the region on the phase diagrams are represented by a blue region of conventional microemulsions. Figures (3-8) represent pseudo-ternary phase diagrams for various surfactants, cosolvents and S mix ratios.

As the ratio of the cosolvent to surfactant increased, so did the area of the nanoemulsion and vice versa for reducing the aforementioned ratio and this particular finding goes with the previously reported findings of other researchers and its approaching consensus-level evidences [11].

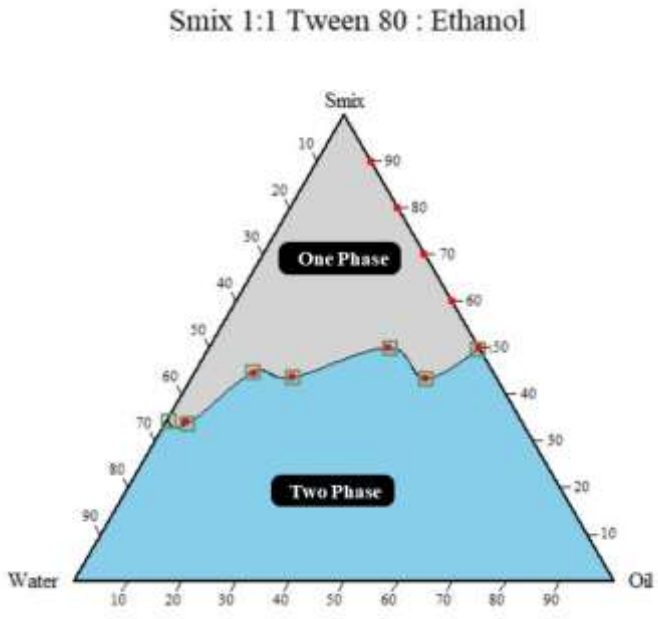


Figure 3: Pseudo ternary phase diagram for oleic oil, $S_{mix}$ (tween 80: ethanol in 1:1 ratio) and water (Fx1-Fx5)

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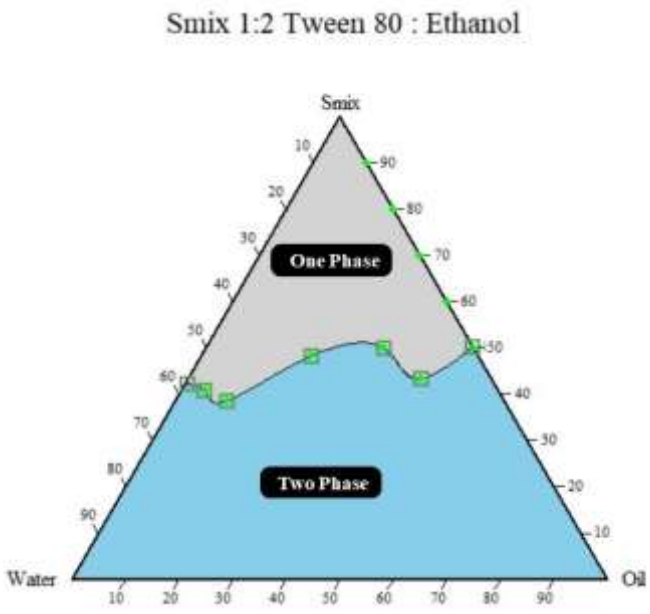


Figure 4: Pseudo ternary phase diagram for oleic oil, $S_{mix}$ (tween 80: ethanol in 1:2 ratio) and water (Fx6-Fx10)

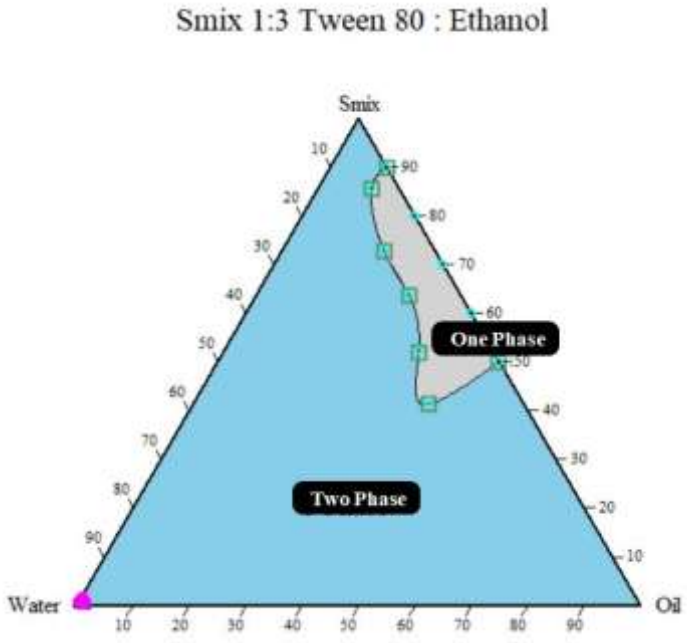


Figure 5: Pseudo ternary phase diagram for oleic oil, $S_{mix}$ (tween 80: ethanol in 1:3 ratio) and water (Fx11-Fx15)

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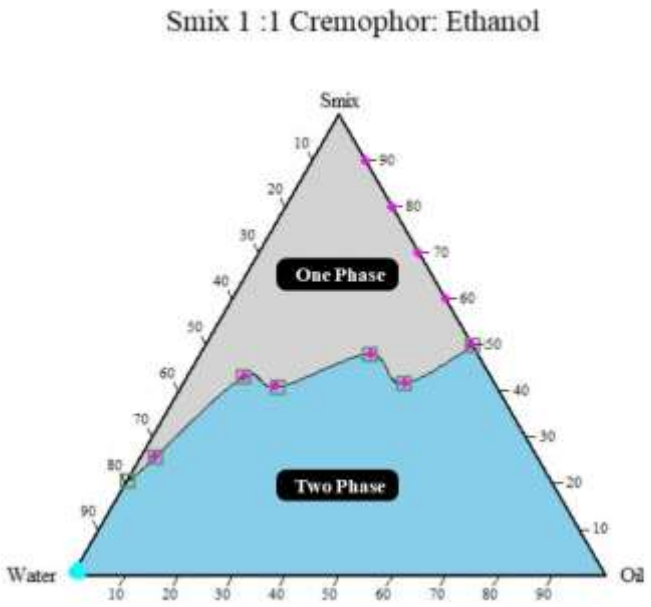


Figure 6: Pseudo ternary phase diagram for oleic oil, $S_{mix}$ (Cremophor: Ethanol in 1:1 ratio) and water (Fx16-Fx20)

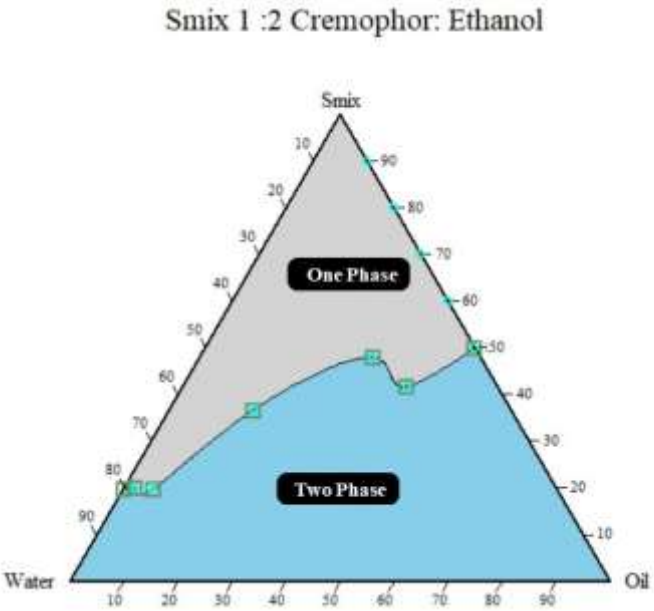


Figure 7: Pseudo ternary phase diagram for oleic oil, $S_{mix}$ (Cremophor: Ethanol in 1:2 ratio) and water (Fx21-Fx25)

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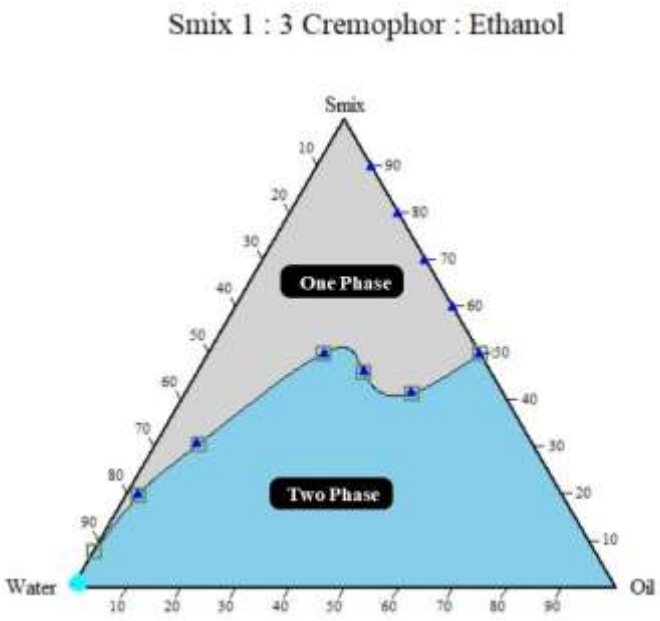


Figure 8: Pseudo ternary phase diagram for oleic oil, $S_{mix}$ (Cremophor: Ethanol in 1:3 ratio) and water (Fx26-Fx30)

From the figures, it's clear that certain formulas and ratios doesn't offer too much in terms of feasibility of nanoemulsion formation. The formulas that allow for higher ratio of water incorporated are particularly interesting and valuable since nanoemulsion in the nasal cavity will be diluted by the mostly aqueous nasal mucous secretion and their ability to be diluted by water without losing their nano range droplet size is of supreme importance to ensure reliable correlation between results gained in vitro and actual conditions that the formulation will face in vivo [12].

Fourteen formulas from these ratios were selected and formulated with the addition of Diazepam and forwarded to droplet size, PDI and zeta potential measurements. Each formula was prepared in the same method described earlier but with dissolving 100mg of Diazepam in the designated volume of oleic acid first.

**2.4. Visual Transparency**

All of the fourteen tested formulas possessed transparent appearance with very minor scattering of light (virtue of their extremely small droplets) which confirm nano-sized droplets and small size range distribution which confers transparency to emulsion preparations.

**2.5. Thermodynamic Stability Studies**

All of the fourteen Diazepam nanoemulsion formulations were successful in all of the three thermodynamic stability tests, proving that they are thermodynamic stable formulations of nano emulsions having none of the creaming, cracking and phase separation which might usually present in the macroemulsions and thus proving the feasibility of these preparation to various storage conditions.

**2.6. Droplet Size and Poly Dispersity Index**

The average droplet size of the tested formulas was in the range of 7.5 to 428nm, as seen in the table 1. It has been observed that as systems incorporate higher percentage of oil, the average droplet size increases, this is due to the expansion of oil droplets of the nanoemulsion by the added extra oil.

Table 1: Average Droplet Size and PDI of Diazepam Nanoemulsions

| F no. | Oil % | $S_{mix}$ type | $S_{mix}$ Ratio | $S_{mix}$ % | Water % | Smix: Oil Ratio | Droplet Size | P.D.I. |
|---|---|---|---|---|---|---|---|---|
| F1 | 10 | Tween 80: Ethanol | 1:1 | 50 | 40 | 9:1 | 12.4 | 0.274 |
| F2 | 10 | Tween 80: Ethanol | 1:1 | 60 | 30 | 9:1 | 166.9 | 0.129 |
| F3 | 10 | Tween 80: Ethanol | 1:2 | 50 | 40 | 9:1 | 7.5 | 0.220 |
| F4 | 10 | Tween 80: Ethanol | 1:2 | 60 | 30 | 9:1 | 41.4 | 0.232 |
| F5 | 10 | Cremephore: Ethanol | 1:1 | 50 | 40 | 9:1 | 65.9 | 0.260 |
| F6 | 10 | Cremephore: Ethanol | 1:1 | 60 | 30 | 9:1 | 12.3 | 0.197 |
| F7 | 10 | Cremephore: Ethanol | 1:2 | 50 | 40 | 9:1 | 148.4 | 0.223 |
| F8 | 10 | Cremephore: Ethanol | 1:2 | 60 | 30 | 9:1 | 110.0 | 0.158 |
| F9 | 20 | Cremephore: Ethanol | 1:2 | 40 | 40 | 8:2 | 428.1 | 0.002 |
| F10 | 10 | Cremephore: Ethanol | 1:3 | 50 | 40 | 9:1 | 259.4 | 0.310 |
| F11 | 10 | Cremephore: Ethanol | 1:3 | 60 | 30 | 9:1 | 373.4 | 0.792 |
| F12 | 20 | Cremephore: Ethanol | 1:3 | 40 | 40 | 8:2 | 279.0 | 0.406 |
| F13 | 5 | Cremephore: Ethanol | 1:2 | 60 | 45 | 0.5:9.5 | 381.3 | 0.321 |
| F14 | 5 | Cremephore: Ethanol | 1:3 | 60 | 45 | 0.5:9.5 | 284.2 | 0.105 |

Another observation was that Tween80 based formulations offered smaller droplets than Cremophor based formula which goes well with previous works of Musa et al [13] and Ammar et al [14] who both reached similar conclusion in their works on chloramphenicol and dorzolamide nano emulsions respectively.

The polydispersity index is a measure of the droplet size distribution. a monodisperse sample has PDI values near zero, but values between 0.1 and 0.3 indicate a tight size distribution, values between 0.1 and 0.4 indicate a moderate size distribution and values larger than 0.4 indicate a wide size distribution. Most of the formulas met the PDI requirements except for formula F11 which unexpectedly was out of the accepted range.

Out of the tested formulas, 5 formulas were selected (F1, F3, F4, F5, AND F6) to be forwarded with the subsequent tests based on having lowest average droplet size and PDI values.

**2.7. Zeta Potential Measurement**

Zeta potential is the major parameter that defines the physical stability of dispersion, where repulsion between similar charges works against the maturing and prevents droplet aggregation with subsequent separation of phase. Zeta potential is regulated by the surfactants type and concentration, system components, product properties, vehicle design, and electrical presence [15].

because the surface-active agent is a nonionic surfactant, and due to diazepam is a weak base with no remarkable ionization at this pH in solution, zeta potential reading should theoretically approach zero but the fact that oil phase is a fatty acid (oleic acid) mainly results in a minor negative surface charge for the droplet. The zeta potential of 5 selected formulas were recorded and values obtained are shown in table 2.

Table 4: Parameters Considered for Selection of Best Formula

| Parameters | Droplet Size | P.D.I. | Zeta potential | pH | % Release in 1st hour |
|---|---|---|---|---|---|
| **Formula 1** | 12.4(acceptable) | 0.274(acceptable) | 5.1- (low) | 5(low) | 57% |
| **Formula 3** | 7.5(acceptable) | 0.220(acceptable) | 5.7-(low) | 5.7(acceptable) | 69% (highest) |
| **Formula 4** | 41.4(acceptable) | 0.232(acceptable) | 5.4-(low) | 4.8(low) | 52% |
| **Formula 5** | 65.9(acceptable) | 0.260(acceptable) | 5.8-(low) | 5.1(borderline) | 33%(lowest) |
| **Formula 6** | 12.3(acceptable) | 0.197(acceptable) | 4.3-(low) | 5.6(acceptable) | 53% |

### 2.8. Percent of Light Transmittance Measurement (%T)

Percent of light transmitted of the selected nano emulsion formulas was measured, versus a blank of distilled water at 650nm throughout the process. The results of the percent of light transmittance of drug-loaded nano emulsions were all in the region of 97–100 percent. This suggests that all formulas of Diazepam nano emulsions appeared clear and transparent.

### 2.9. Dye Test

Methyl orange is an azo dye freely miscible with water. After adding methyl orange dye drop by drop to five formulations, it mixed with the external aqueous phase and was miscible homogeneously with no cloudiness or aggregates across all five formulations.

### 2.10. pH Measurement

The pH of 5 nano emulsions (F1, F3. F4, F5 and F6) has been measured using pH meter. The pH values were ranged from 4.8 to 5.7 as shown in table 2. These values place these formulations at the lower end of the acceptable pH range of nasal formulations which is 5.1 – 8.1 and thus they may cause change in physiological pH of nasal cavity and irritation ensues [16].

### 2.11. In Vitro Drug Release Study

Figure 9 displays the different release profiles for Diazepam nano emulsions formulas F1, F3, F4, F5 and F6 within one hour from commencing release test from a dialysis membrane bag with a 12000 Dalton molecular weight cut-off, Dialysis bags were washed with deionized water and soaked overnight in phosphate buffer saline of pH 6.4 corresponding to the pH of nasal fluid.

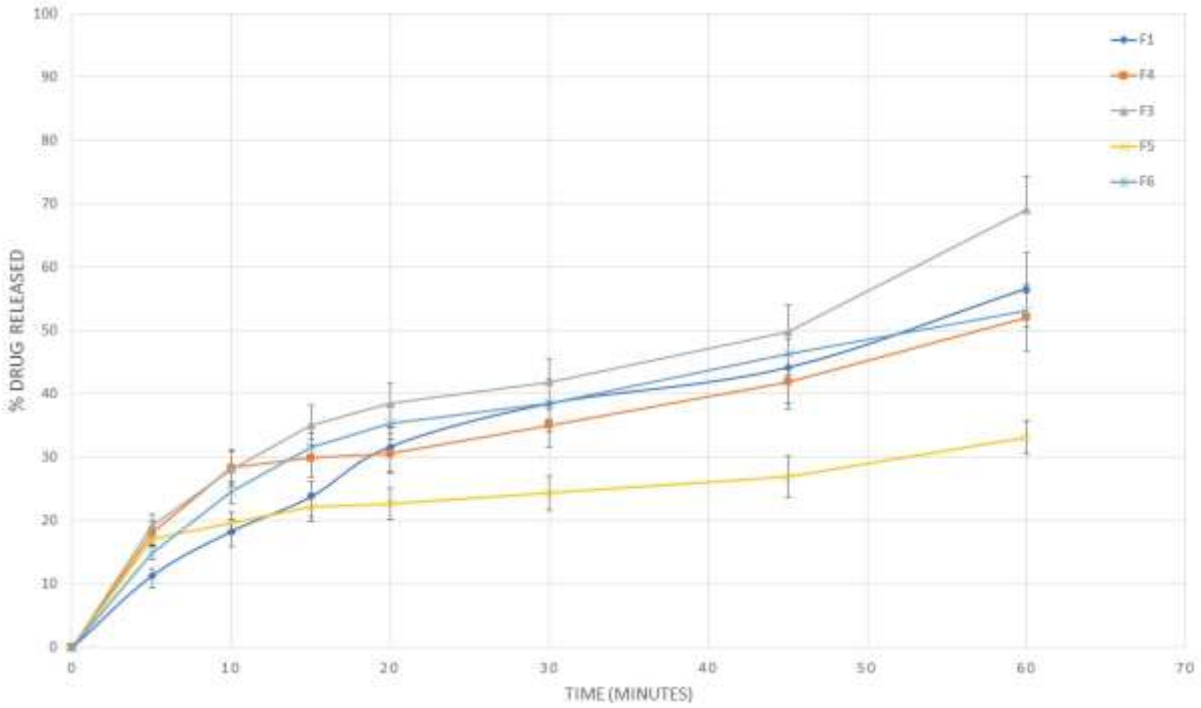


Figure 9: release profiles of five Diazepam nanoemulsion formulas

The test can be continued for more than one hour in lab conditions but the actual in vivo conditions of nasal cavity will virtually clear more than 93% of the administered formula within one hour [17] and further continuation of the release test will be in reality of no significance.

The highest release was seen in F4 and it reached 70% in one hour which is below the favorable 100% release figure but it was unfortunately expected from a drug that is practically insoluble in water and is completely unionized at the nasal cavity pH of 6.4.

It can be concluded from the results of release tests that Tween 80 based formulas have faster and more complete release in the first hour than Cremophor based formulas (57%, 52% and 69% versus 33% and 53% respectively) which could be owned to the fact that Tween 80 based formulas have smaller droplet size which contribute to faster partition of drug and release from the emulsion and also to Tween 80 having higher HLB value than Cremophor and consequently higher hydrophilicity and reduction in interfacial tension between the oil and the aqueous phase [18].

Formulas with higher surfactant to cosolvent ratios of 1:2 (F3 & F4) had faster initial release from formulas with lower ratios (F1, F5 & F6) which is consistent with what has been found with similar previous research. It can be postulated to have connection with the effect of cosolvent in Nano emulsion systems, which diminishes the interfacial tension and further fluidize the interface between oil droplets and water/$S_{mix}$ phase of the interface by mobilizing the hydrocarbon tail of the surfactant [19].

**2.12. Selection of Best Diazepam Formula**

Out of the 5 formulations of Diazepam Nano emulsions tested in regards of release profile, pH of formula, droplet size, poly dispersity index values and zeta potential values. All offered similar zeta potential values and good droplet size and size distribution. However, differences in percent released in the first hour and acceptable pH gave formula 3 a head start in drug release.

The pH of formula 1 and formula 4 were below the acceptable range and would probably lead to irritation of mucous membranes causing increased mucosal production and ciliary clearance leaving the formulation with shorter time for release and absorption.

Formula 3 Diazepam nanoemulsion with $S_{mix}$ combination (Tween 80: ethanol ratio of 1:2): Oil: water (50: 10: 40) ratio was selected as the best formula among tested formulas, since it collectively outperformed other formulas in terms of possessing good droplet size range (7.5 nm), good PDI (0.22), good percent transmittance (98.5), and highest release percentage of Diazepam from the formula in the first hour (69%).

## 3. CONCLUSION

The main conclusions of the study should be presented in a short Conclusions section, which stands alone. You should explain whether your findings supported your hypothesis in this section. Avoid using references in conclusion section.

## 4. MATERIALS AND METHODS

### 4.1. Construction of Calibration Curves:

Calibration curves of Diazepam in ethanol and phosphate buffer pH 6.4 were established by diluting the stock solution in a 1:20 ratio then preparing a series of dilutions of the drug by transferring (0.062, 0.125, 0.25, 0.5 and 1mL) from a stock solution of 1mg/5mL potency to 5mL volumetric plastic tubes and diluting up to the 5mL. The UV absorbances of these diluted drug solutions were measured at λmax of 242nm and plotted against sample concentrations to build an absorbance versus concentration calibration curve. The test was done in triplicate (n=3). The $R^2$ value and calibration curve equation was calculated.

### 4.2. Determination of Diazepam Solubility in Surfactants and Cosurfactants

The determination of Diazepam solubility in different surfactants and cosolvents (Tween 80, Cremophor, and ethanol) were estimated as follows: An excess amount of Diazepam powder was accurately weighted and added to 5ml of each liquid media then placed in tightly capped glass tubes with metal screws, vortexed for few minutes to ensure homogeneity then put on a water bath shaker at low RPM for 48 hours at 25° C to ensure reaching equilibrium then the samples have been filtered through a membrane filter (0.45µm) and appropriately diluted in ethanol. The solubilities were determined spectrophotometrically at λmax of 242nm with the test done in triplicate (n=3).

### 4.3. Construction of Ternary Phase Diagrams:

The ternary phase diagrams containing oil, surfactant, cosolvent and water were developed by the spontaneous emulsification method (aqueous titration method).

The pseudo-ternary phase diagrams were designed to evaluate different surfactant/cosurfactant mixture ($S_{mix}$) with Various $S_{mix}$ ratios (1:1, 1:2, and 1:3) were tried to achieve the required HLB value (8–18) for O/W emulsion formation. Various ratios of oil to selected emulsifiers ($S_{mix}$) (ranging between 5:5 to 9:1) were prepared to determine the emulsion area boundaries.

The mixtures of $S_{mix}$ and oil were titrated with water separately by adding 0.5 mL of water (drop by drop) at room temperature under gentle stirring (~500 rpm), and was optically observed. The water amount at which the transition occurs from transparency to turbidity end point was recorded from measurements of the volume. Table 3 show the thirty initial experimental formulas that has been prepared just for the quantification of the amount of water required for transition and were forwarded for phase diagram visualization. Phase diagrams were visualized using CHEMIX School Ver. 3.50 software (MN, USA) [20].

Table 3: Experimental Formulations of Nanoemulsions for Pseudo-ternary Diagrams

| $S_{mix}$ Components | $S_{mix}$ Ratio | Formula No. | $S_{mix}$ : Oil Ratio | |
|---|---|---|---|---|
| | | | **$S_{mix}$** | **Oil** |
| Tween 80: Ethanol | 1:1 | Fx1 | 50 | 50 |

| | | | | |
|---|---|---|---|---|
| | | Fx2 | 60 | 40 |
| | | Fx3 | 70 | 30 |
| | | Fx4 | 80 | 20 |
| | | Fx5 | 90 | 10 |
| Tween 80: Ethanol | 1:2 | Fx6 | 50 | 50 |
| | | Fx7 | 60 | 40 |
| | | Fx8 | 70 | 30 |
| | | Fx9 | 80 | 20 |
| | | Fx10 | 90 | 10 |
| Tween 80: Ethanol | 1:3 | Fx11 | 50 | 50 |
| | | Fx12 | 60 | 40 |
| | | Fx13 | 70 | 30 |
| | | Fx14 | 80 | 20 |
| | | Fx15 | 90 | 10 |
| Cremephore: Ethanol | 1:1 | Fx16 | 50 | 50 |
| | | Fx17 | 60 | 40 |
| | | Fx18 | 70 | 30 |
| | | Fx19 | 80 | 20 |
| | | Fx20 | 90 | 10 |
| Cremephore: Ethanol | 1:2 | Fx21 | 50 | 50 |
| | | Fx22 | 60 | 40 |
| | | Fx23 | 70 | 30 |
| | | Fx24 | 80 | 20 |
| | | Fx25 | 90 | 10 |
| Cremephore: Ethanol | 1:3 | Fx26 | 50 | 50 |
| | | Fx27 | 60 | 40 |
| | | Fx28 | 70 | 30 |
| | | Fx29 | 80 | 20 |
| | | Fx30 | 90 | 10 |

The larger the area of one phase nanoemulsion in each phase diagram (i.e., having greater hydration capacity) is selected as the best emulsion composition and selected for further study.

### 4.4. Preparation of Diazepam Nanoemulsions

The best 14 formulas of $S_{mix}$ and Oil combinations with regard to area of phase diagram obtained were selected, drug was introduced to Oil & $S_{mix}$ and were further proceeded to droplet size analysis. Diazepam nanoemulsion was prepared by using Tween 80 and cremephore EL as surfactant and ethanol as co-surfactant in different ratios (surfactant: co surfactant) (1:1, 1:2, and 1:3) with oleic acid as the oil phase in the combinations mentioned in Table 4. The drug was combined with each of these formulations at the dose of 10mg of Diazepam per one milliliter of the respective nanoemulsion. Diazepam was accurately weighted and dissolved in a respective amount of oil then the respective quantity of $S_{mix}$ added for oil loaded drug, after that the whole mixture was blended by a vortex mixer for 5 minutes, at the speed of 100rpm. Then the aqueous phase (deionized distil water) was added gradually to obtain clear O/W nanoemulsion.

Table 4: Diazepam Nanoemulsions Formulations

| F no. | Oil % | $S_{mix}$ type | $S_{mix}$ Ratio | $S_{mix}$ % | Water % | Drug w/v% | $S_{mix}$: Oil Ratio |
|---|---|---|---|---|---|---|---|
| F1 | 10 | Tween 80: Ethanol | 1:1 | 50 | 40 | 1 | 9:1 |
| F2 | 10 | Tween 80: Ethanol | 1:1 | 60 | 30 | 1 | 9:1 |
| F3 | 10 | Tween 80: Ethanol | 1:2 | 50 | 40 | 1 | 9:1 |
| F4 | 10 | Tween 80: Ethanol | 1:2 | 60 | 30 | 1 | 9:1 |
| F5 | 10 | Cremephore: Ethanol | 1:1 | 50 | 40 | 1 | 9:1 |
| F6 | 10 | Cremephore: Ethanol | 1:1 | 60 | 30 | 1 | 9:1 |
| F7 | 10 | Cremephore: Ethanol | 1:2 | 50 | 40 | 1 | 9:1 |
| F8 | 10 | Cremephore: Ethanol | 1:2 | 60 | 30 | 1 | 9:1 |
| F9 | 20 | Cremephore: Ethanol | 1:2 | 40 | 40 | 1 | 8:2 |
| F10 | 10 | Cremephore: Ethanol | 1:3 | 50 | 40 | 1 | 9:1 |
| F11 | 10 | Cremephore: Ethanol | 1:3 | 60 | 30 | 1 | 9:1 |
| F12 | 20 | Cremephore: Ethanol | 1:3 | 40 | 40 | 1 | 8:2 |
| F13 | 5 | Cremephore: Ethanol | 1:2 | 60 | 45 | 1 | 0.5:9.5 |
| F14 | 5 | Cremephore: Ethanol | 1:3 | 60 | 45 | 1 | 0.5:9.5 |

### 4.5. In Vitro Evaluation of Diazepam Formulas (F1-F14)

#### 4.5.1. Visual Transparency:

Optical transparency for 14 Diazepam formulas were determined by visual inspection the 14 formulas in transparent, clear glass vials under adequately bright light source and against white illuminated background [21].

#### 4.5.2. Droplet Size, Poly Dispersity Index and Zeta Potential Measurement:

Mean droplet size, zeta potential (droplet surface charge) and polydispersity index size range for 14 Diazepam formulas were determined by using dynamic light scattering technique (Zetasizer Nano ZS) where light scattering fluctuations were analyzed due to Brownian motion of droplet in their vehicles.

The high viscosity of oleic acid and emulsifiers might interfere with accurate size determination and was reduced by diluting 0.1 mL of each formula with distilled water (50 mL) under gentle agitation to reduce the errors caused by multiple scattering effect that is known of dense oily emulsions. One milliliter of diluted resultant nanoemulsion was injected into folded capillary zeta cell and the light scattering was measured at 25°C at 90° angle.

Polydispersity index (PDI) is used to assess the uniformity of droplet sizes in a nanosuspension, nanoemulsion or a nanogel. The higher a polydispersity value indicate lower uniformity of droplets size of that preparation.

The average droplet size, zeta potential and polydispersity index values were recorded for each formula in triplicate (n=3) and the best 5 formulas were forwarded for further testing.

### 4.6. Thermodynamic Stability Studies

All 14 formulations were subjected to the various thermodynamic stability tests of centrifugation, heating-cooling cycle and freeze-thaw cycle to assessing capability to overcome different ambient conditions that may be faced during preparation and storage.

### 4.7. Centrifugation Test

All 14 formulation were centrifuged at 3500 rpm for half an hour, observed for phase separation, creaming, coalescence and cracking. Stable formulations were forwarded to the heating-cooling cycle.

### 4.8. Heating-Cooling Cycle

Stability of nanoemulsion by rapid variation of temperature were measuring cycling through repeated cycles of high and low temperatures then observing phase changes mentioned in Centrifugation test above. Six cycles between refrigerator temperature 4°C and high temperature of 45°C with storage at each temperature for not less than 48hr. Formulations, which are stable at these temperatures, were forwarded to freeze-thaw cycle.

### 4.9. Freeze-Thaw Cycle

Three freeze-thaw cycles between minus 21°C and room temperature of 25°C with storage at each temperature for not less than 48hr was carried out for all 14 formulas. Formulations which passed these thermodynamic stress tests were proceeded to size analysis studies.

### 4.10. Percent of Light Transmittance Measurement (*%T*)

This test is used to verify the translucence of the prepared formulas. This carried out by taking 2 mL of each formula and recording the absorbance at 650 nm using UV-VIS spectrophotometer and distilled water as blank. The transmittance percentage (%T) was calculated using the equation;

$A = 2 - \log \%T$ Where; A: absorbance, %T: transmittance percentage

### 4.11. Dye Test

A water-soluble dye (methyl orange was used) is mixed with each formula. If a dye mixed with the formula gave a homogenous tainting without cloudiness or precipitation, it indicates that the continuous phase is water and the nanoemulsion under test is indeed of O/W type.

### 4.12. pH Measurement

The maintenance of the pH of nasal cavity is very important within its physiological limits of 5.5 – 6.5 mainly to avoid irritation of the nasal mucosa, to maintain physiological ciliary movement and to prevent the growth of pathogenic bacteria [22].

The pH of all prepared formulas was measured using a pH meter. The formulas were as a 30-ml sample placed in a 50-ml graduated beaker at room temperature and the measurements were repeated in triplicates (n=3).

### 4.13. In Vitro Drug Release Study

Diazepam release was followed in vitro using a modified membrane dialysis technique for 5 formulations (F1, F3, F4, F5, F6). The precise volume that contained 2.5mg of the chosen formulas was transferred into the dialysis membrane with a 12000 Dalton molecular weight cut-off), Dialysis bags were soaked overnight in phosphate buffer of pH 6.4 (normal of pH of nasal fluid. The membrane was then closed from both ends by sealing clamps and then was immersed in a glass cylinder containing 500 mL phosphate buffer (pH 6.4), tied to the paddle fins with a light thread and allowed to rotate with a magnetic stirring at a speed of 50 rpm at preconditioned and maintained 37°C. This was tried after failure to track the release of a 10mg containing volume of samples since sink conditions for a practically insoluble drug was difficult to achieve in a 500ml of buffered dissolution media. A series of 2mL aliquots of medium were withdrawn at interval of 5, 10, 15, 20, 30, 45 and 60min and immediately replaced with 2ml of fresh phosphate buffer pH 6.4. UV-Visible spectrophotometry was used to determine the absorption of the dialysis solution at various times at 245.5 nm. The test was done in triplicate (n=3).

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