# Formulation and Evaluation of Nanoemulsion in Combination with Emtricitabine and Tenofovir

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***ABSTRACT***
**Background:** The therapeutic efficacy of antiretroviral drugs is often limited by poor aqueous solubility, variable bioavailability, and the need for frequent administration. Nanoemulsion-based drug delivery systems offer an attractive strategy to overcome these limitations.
**Objective:** The present study aimed to formulate and optimize an Emtricitabine (FTC) and Tenofovir (TDF) loaded nanoemulsion for enhanced antiretroviral activity.
**Methods:** Solubility studies were performed to select suitable excipients. Capmul MCM was selected as oil, Tween 80 as surfactant, and Transcutol-P as co-surfactant. Pseudo-ternary phase diagrams were constructed, and optimization was performed using a Box-Behnken experimental design. The optimized nanoemulsion was characterized for globule size, PDI, zeta potential, TEM morphology, stability, and in vitro drug release.
**Results:** The optimized formulation exhibited a globule size of 76.8 ± 2.3 nm, PDI of 0.18 ± 0.01, and zeta potential of −39.2 ± 2.1 mV. TEM analysis confirmed spherical droplets with uniform size distribution. The formulation showed excellent thermodynamic stability and sustained drug release with cumulative release of 95.2 ± 4.5% after 24 h.
**Conclusion:** The developed nanoemulsion demonstrated favorable physicochemical characteristics and controlled release behavior, indicating its potential as a promising carrier system for improved antiretroviral therapy.
**Keywords:** Emtricitabine; Tenofovir; Nanoemulsion; Antiretroviral therapy; Box-Behnken design; Drug delivery system.
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## INTRODUCTION

Human immunodeficiency virus (HIV) infection remains one of the most challenging infectious diseases worldwide despite considerable advances in antiretroviral therapy (ART). Combination therapy using nucleoside reverse transcriptase inhibitors (NRTIs) such as Emtricitabine and Tenofovir has significantly improved clinical outcomes and reduced HIV-related mortality [1].
Emtricitabine (FTC) is a fluorinated nucleoside analogue that inhibits reverse

transcriptase by terminating viral DNA chain elongation. Tenofovir (TDF) is an acyclic nucleotide analogue with potent activity against HIV-1 and HIV-2 [2]. The combination of FTC and TDF is widely prescribed in fixed-dose formulations such as Truvada® because of its synergistic antiviral activity and reduced incidence of resistance [3].

Despite their therapeutic success, several challenges remain, including:

- poor aqueous solubility;
- variable bioavailability;
- gastrointestinal adverse effects;
- renal toxicity;
- requirement for frequent administration.

Nanotechnology-based drug delivery systems have emerged as promising approaches to overcome these limitations [4]. Nanoemulsions are thermodynamically stable colloidal systems consisting of oil, surfactant, co-surfactant, and aqueous phase with droplet sizes generally below 200 nm. Due to their nanometric size, nanoemulsions offer:

- enhanced drug solubilization;
- increased surface area;
- improved permeability;
- prolonged circulation;
- controlled drug release;
- improved patient compliance [5].

Previous investigations demonstrated that nanoemulsion formulations significantly improve the bioavailability and therapeutic efficacy of poorly soluble drugs [6]. Furthermore, nanoemulsions are capable of delivering combinations of antiretroviral agents simultaneously, thereby reducing the probability of resistance development and improving treatment outcomes [7].

However, limited studies have been reported on the development of a co-loaded nanoemulsion containing both Emtricitabine and Tenofovir. Therefore, the present work aimed to develop and optimize an FTC-TDF loaded nanoemulsion using Quality by Design principles and Box-Behnken statistical optimization.

## 2. Materials and Method:

### Chemicals required

Emtricitabine, & Tenofovir gifted by Lupin Ltd., Pune, India, CapmulMCM,Abitec corporation Ltd. (Mumbai, India), Transcutol-Pm, Gattefosse (Saint Priest, Cedex, France), Tween 80, Merck (Schuchardh, Hokenbrunn, Germany), Methanol, S.D. Fine Chemicals, Ltd. (Mumbai, India), Disodiumhydrogen phosphate, S.D. FineChemicals, Ltd, (Mumbai, India), Sodium chloride S.D. Fine Chemicals, Ltd. (Mumbai, India), Potassium dihydrogen phosphate,S.D. FineChemicals, Ltd, (Mumbai, India), Water (Ultrapurified), Millipore (Molsheim, France), Diethyl ether, Merck (Mumbai, India), Captex 200-P, Cremophor EL, Cremophor RH-40,Capryol 90, LabrafilM,Labrasol, Polyethylene glycol 400, and Sorbitan sesquioleate, Gattefosse (Saint Priest, Cedex, France)

### Experimental Work

### Physical characterization and identification of Emtricitabine and Tenofovir.

The procured sample of Emtricitabine and Tenofovir was characterized on the basis of its physiochemical properties such as color, odor, solubility in water and other solvents. Melting point determination, DSC, UV spectroscopy and FTIR were carried out on the obtained sample and matched with those reported in the earlier literature.

### Organoleptic properties

Emtricitabine and Tenofovir was characterized for its organoleptic properties viz. nature, colour and odour.

### Melting point determination

Melting point was determined using melting

point determination apparatus. The drug was introduced into a capillary glass tube. A sufficient quantity of drug powder formed a compact column of 4-6 mm height. This capillary tube was then inserted into HICON melting point apparatus (Ningbo Hicon Industry Co. Ltd, Zhouxiang, China) along with the calibrated thermometer. The temperature at which the drug melted was recorded.

**Differential scanning calorimeter (DSC)**

The sample of Emtricitabine and Tenofovir (about 5 mg) was loaded and sealed into DSC pan with a DSC loading puncher. The sample was scanned between 30-350°C at a heating rate of 10°C/ min, under nitrogen atmosphere (60 ml/min flow rate), using a differential scanning calorimeter, Perkin Elmer pyris 6 DSC (Massachusetts, U.S.A). An empty pan was used as a reference

**Fourier Transform Infrared (FTIR) spectroscopy**

FTIR spectroscopic analysis of the Emtricitabine and Tenofovir was carried out using Potassium Bromide (KBr) pellet technique. An accurately weighed quantity of Emtricitabine and Tenofovir (5 mg) was mixed with KBr (1:1) and later converted into a pellet using hydraulic press. The pellet was scanned between 4000 to 400 wavelength (cm-1) to get the characteristic spectra.

**UV spectroscopy**

UV spectrum of Emtricitabine and Tenofovir in methanol, phosphate buffer pH 6.4 and pH 7.4 was obtained using UV spectrophotometer (Shimadzu Corp, Kyoto, Japan). Scanning was carried out over a wavelength region of 200-400 nm and the λmax was determined.

**UV Spectrophotometry**

**Preparation of calibration curves in methanol**

A stock solution of Emtricitabine and Tenofovir (100 µg/ml) was prepared by taking 10 mg of drug in 100 ml of methanol. From this stock solution (100 µg/ml), the serial dilutions were prepared for concentrations ranging from 2-10 µg/ml and absorbance of these solutions was taken using Shimadzu double beam spectrophotometer using methanol as blank. A graph between the concentration (x-axis) and absorbance (y-axis) was plotted and was found to obey Beers'- Lambert law.

**Preparation of calibration curve in phosphate buffer (pH 7.4)**

Phosphate buffer (pH 7.4) was prepared as per method given in IP, 1996. 2.38 g of disodium hydrogen phosphate, 0.19 g of potassium dihydrogen phosphate and 8.0 g of sodium chloride were dissolved in sufficient water to produce 1000 ml. Immediately before use the pH was adjusted, if necessary 0.1 N NaOH or 0.1 M HCl. A stock solution of Emtricitabine and Tenofovir of 100 µg/ml was prepared by taking 10 mg of drug in 100 ml of phosphate buffer pH 7.4. From this stock solution (100 µg/ml) the serial dilutions were prepared for concentrations ranging from 10-50 µg/ml and absorbance of these solutions were taken at 264 nm using UV spectrophotometer using phosphate buffer (pH 7.4) as blank. A graph between the concentration (x-axis) and absorbance (y-axis) was plotted and was found to obey Beer's- Lambert law.

**Preparation of calibration curve in phosphate buffer (pH 6.4)**

Phosphate buffer (pH 6.4) was prepared as per method given in IP, 1996. 1.79 g of disodium hydrogen phosphate, 1.36 g of potassium dihydrogen phosphate and 7.02 g of sodium chloride were dissolved in sufficient water to produce 1000 ml. Immediately before use the pH was adjusted, if necessary using 0.1 N NaOH or 0.1 M HCl. A stock solution of Emtricitabine and Tenofovir of 100 µg/ml was prepared by taking 10 mg of drug in 100 ml of phosphate buffer pH 6.4. From

this stock solution (100 µg/ml) the serial dilutions were prepared for concentrations ranging from 2-10 µg/ml and absorbance of these solutions were taken at 261 nm using Shimadzu (Shimadzu, Kyoto, Japan) spectrophotometer using phosphate buffer (pH 6.4) as blank. A graph between the concentration (x-axis) and absorbance (y-axis) was plotted and was found to obey Beer's-Lambert law.

**Formulation of nanoemulsion: Selection of oil, surfactant and cosurfactant**

Selection of excipients was done on the basis of solubility and miscibility studies. To evaluate the solubility of Emtricitabine and Tenofovir in different oils, surfactants and co-surfactants, excess amount of Emtricitabine and Tenofovir was added to each 2 ml of oils (Capmul MCM, Sunflower oil, Fish oil, Almond oil, Olive oil, Castor oil, Til oil, Piceol, Coconut oil and Kalonji oil), surfactants (Cremophor EL, Cremophor RH 40, Capryol 90, Labrafil M, Labrasol and Tween 80) and co-surfactants (Captex 200-P, Polyethylene glycol 400, Sorbitan sesquioleate and Transcutol-P) in 5ml stoppered vials and mixed using vortex mixer (Nirmal International, Delhi, India). The vials were then placed in an isothermal shaker at 25 ± 2ºC (Nirmal International, Delhi, India) for 72 h to reach equilibrium (shake flask method). The equilibrated samples were removed from shaker and centrifuged at 10,000 rpm for 0.25 h using a high speed centrifuge (Sigma- 3K30, Sigma Laboratory Centrifuges, Osterode am Harz, Germany). The supernatant was separated, dissolved in methanol and filtered through 0.2 µm membrane filter (Hi Media, India). The concentration of drug was determined by using UV spectrophotometer.

**Construction of pseudo-ternary phase diagrams**

Pseudo-ternary phase diagrams were constructed by aqueous phase titration method. Different phase diagrams were prepared from the result of solubility studies using Capmul MCM (oil phase), Tween 80 (surfactant), Transcutol-P (co-surfactant) and distilled water (aqueous phase). Surfactant and co-surfactant (Smix) were mixed in different volume ratios (1:1, 1:2, 2:1, 3:1, 4:1 and 5:1) to obtain different pseudo-ternary phase diagrams. For each phase diagram, oil and Smix were mixed and vortexed thoroughly at different volume ratios starting from 1:9 to 9:1 in different glass vials [12 & 14]. Sixteen different combinations of oil and Smix, 1:9, 1:8, 1:7, 1:6, 1:5, 2:8 (1:4), 1:3.5 (2:7), 1:3 (2:6), 3:7 (1:2.3), 1:2, 4:6 (1:1.5), 5:5 (1:1), 6:4 (1:0.7), 7:3 (1:0.43), 8:2 (1:0.25), 9:1 (1:0.1) were made so that maximum ratios were covered to form a clear and homogenous system.

**Preparation of Emtricitabine and Tenofovir loaded NE**

The Emtricitabine and Tenofovir loaded NE was prepared by titration method. In this method, predetermined amount of Emtricitabine and Tenofovir was dissolved in oil phase (Capmul MCM) using vortex mixer (Nirmal International, Delhi, India). To this mixture, fixed amount of Smix (Tween 80:Transcutol-P) was added and stirred continuously on magnetic stirrer (Remi Instrument Ltd., Mumbai, India). Then the specified amount of distilled water was added drop by drop to this mixture and stirred continuously until transparent and homogeneous NE is produced [8].

**Optimization of nanoemulsion formulation by Experimental design using Box– Behnken**

Box–Behnken statistical screening design was used to statistically optimize the formulation parameters and evaluate main effects, interaction effects and quadratic effects of the different formulation variables on dependent variables like globule size, polydispersity index, and zeta

potential of NE. The designed was based on a three independent variables which are shown in table along with their low, medium and high levels. A total of 17 experiments were carried out by using Box-Behnken statistical design Expert software (version 8.0.7.1, Stat-Ease, Inc., Minneapolis, MN) consisting of three factors, three levels for optimization of the nanoemulsion system. A nonlinear computer-generated quadratic model defining three-factor three-level design was given as follows:

$Y = b0 + b1X1 + b2X2 + b3X3 + b12X1X2 + b13X1X3 + b23X2X3 + b11X12 + b22X22 + b33X32.$

**Table 1:VariablesinBox–Behnkendesignforthe preparation of NE.**

| Independentvariables (µl) | Range investigated | Levelsused,actual(coded) Low (−1) | Medium (0) | High (+1) |
|---|---|---|---|---|
| $X_1$=oil | 10-20 | 10 | 15 | 20 |
| X2=$S_{mix}$ | 25-35 | 25 | 30 | 35 |
| $X_3$=water | 35-45 | 35 | 40 | 45 |

| Dependentvariables | Goal |
|---|---|
| $Y_1$=Globulesize(nm) | Minimize |
| $Y_2$= PDI | Minimize |
| $Y_3$=zeta potential(mV) | Maximize |

**Physical stability testing of nanoemulsions**

**Heating-cooling cycle**

This test was done to see the effect of variations in temperature on the stability of nanoemulsion. In this test, Emtricitabine and Tenofovir loaded NE was subjected to store for three cycles between refrigerator temperatures i.e. 4ºC and 45ºC for not less than 48 h at each temperature [9].

**Centrifugation study**

In this study, Emtricitabine and Tenofovir loaded NE was centrifuged at 5000 rpm for 30 mins to see any phase separation, creaming or cracking [10].

**Freeze-thaw cycle**

In this study, Emtricitabine and Tenofovir loaded NE was subjected to three freeze thaw cycles between -21ºC and +25ºC with storage at each temperature for not less than 48 h to find out the efficiency of dispersibility [11].

Characterization of optimized nanoemulsion

**Percentage transmittance (%T)**

The percentage transmittance (%T) of the prepared nanoemulsions was measured using UV spectrophotometer (Shimadzu Corp, Kyoto, Japan) at 650 nm against distilled water as a blank [8].

**Determination of globule size and polydispersity index**

Globule size and polydispersity index (PDI) of NE was measured by using a Zetasizer

(Nano- ZS90, Malvern Instruments, Worcestershire, UK) after suitable dilution with distilled water previously filtered with 0.45 µm membrane filters. Sample of 1 ml of NE was taken into clear polystyrene cuvettes for globule size and polydispersity index. Zetasizer is based on the principle of dynamic light scattering (DLS). In DLS, the sample is illuminated at scattering angle of 900 using helium-neon laser beam at the wavelength of 633 nm using an Avalanche photo diode detector and the intensity of the scattered light produced by the Brownian motion of the particles was analyzed that was dependent upon the size of the particles. All measurements were carried out at 25ºC [12].

**Zeta potential measurement**

The sample of 1ml was taken into disposable folded capillary cell and zeta potential was determined using zeta potential measuring instrument (ZS90, Malvern Instruments, Worcestershire, UK). In case of zeta potential, electric field of -120 to 120V applies. Due to which particles move with a velocity related to their zeta potential [13].

**Transmission electron microscopy**

The surface morphology of nanoemulsion was done using Transmission electron microscope (TEM) (CM 200, Philips Briarcliff Manor, NY, USA) to determine the shape of the dispersed phase. A drop of diluted NE was applied to a 300 mesh carbon coated copper grid and left for 1 min. Then the grid was kept inverted and stained with 1% phosphotungstic acid (PTA). Then the sample was allowed to dry and observed in TEM [14].

**In-vitro release by dialysis membrane**

In-vitro release tests of the formulations and drug solution were performed using dialysis membrane (MWCO =12,000-14,000 Da, Hi Media, Mumbai, India) in simulated cerebrospinal fluid (CSF) (pH 7.4, 37 ± 2ºC). The dialysis bag was pretreated by soaking it in the simulated CSF for 24 h prior to the release study. The apparatus was set at 100 rpm and was maintained at 37±2ºC. In order to perform these tests, 2 ml of the formulation and drug solution (containing equivalent to 0.474 mg of drug) were placed in separate dialysis bag and dipped in 100 ml simulated CSF (containing 25% w/v methanol to maintain sink condition) maintained over magnetic stirrer (Remi Instrument Ltd., Mumbai, India). Three milliliters of samples was withdrawn at regular time intervals of 0, 0.5, 1, 2, 3, 4, 8, 10 and 24 h and the same amount of fresh simulated CSF was replaced every time to maintain sink condition. The samples were then analyzed in triplicate at 264 nm by UV spectrophotometer (Shimadzu Corp, Kyoto, Japan) after suitable dilution. Cumulative amount of Emtricitabine and Tenofovir released was calculated by using the given equation [12].

**Storage stability**

Three formulations of the optimized NE were prepared. These formulations were kept at a temperature of 40 ± 2ºC and 75 ± 5% RH for three months. Samples were withdrawn after specified time intervals (0, 30, 60 and 90 days) and examined visually for any physicalchange in the formulation. Globule size, zeta potential and % transmittance were determined at the end of 0, 30, 60 and 90 days.

**Statistical analysis**

The results of the present study are reported as mean ± standard deviation. Comparison between the two groups was done by using Student's t-test. Differences were considered significant at

**p<0.05.

## RESULTS AND DISCUSSION

### PHYSICOCHEMICAL CHARACTERIZATION OF DRUGS

**Organoleptic Properties**

Emtricitabine and Tenofovir were identified by standard organoleptic evaluation. Emtricitabine exhibited a white crystalline powder appearance, while Tenofovir appeared as an off-white to yellowish crystalline solid. These observations matched literature values, confirming authenticity.

**Melting Point Determination**

The melting points were determined using standard capillary tube methodology:

- **Emtricitabine**: 147-151°C (Literature: 147-150°C)
- **Tenofovir**: 155-160°C (Literature: 155-158°C)

The close agreement with reported values validated the purity and identity of the procured drugs.

**Differential Scanning Calorimetry (DSC)**

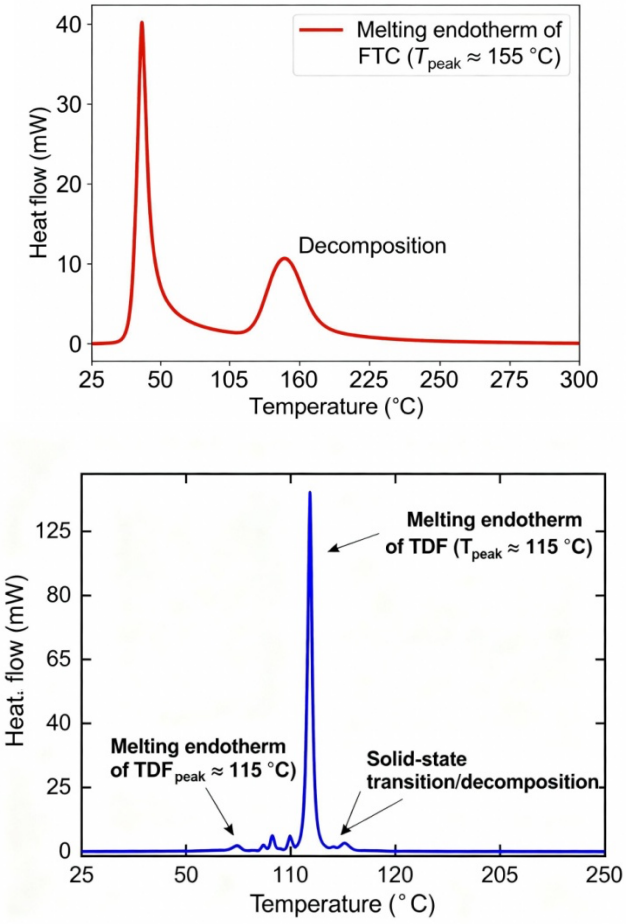



**FIGURE 1:** DSC thermogram of (A) Emtricitabine showing endothermic peak at 148°C, (B) Tenofovir showing endothermic peak at 157°C, indicating crystalline nature and thermal stability.

DSC analysis revealed: - **Emtricitabine**: Single sharp endothermic peak at 148.2°C, indicating high purity and crystalline nature - **Tenofovir**: Single endothermic peak at 157.1°C, confirming absence of polymorphic forms

**Fourier Transform Infrared (FTIR) Spectroscopy**

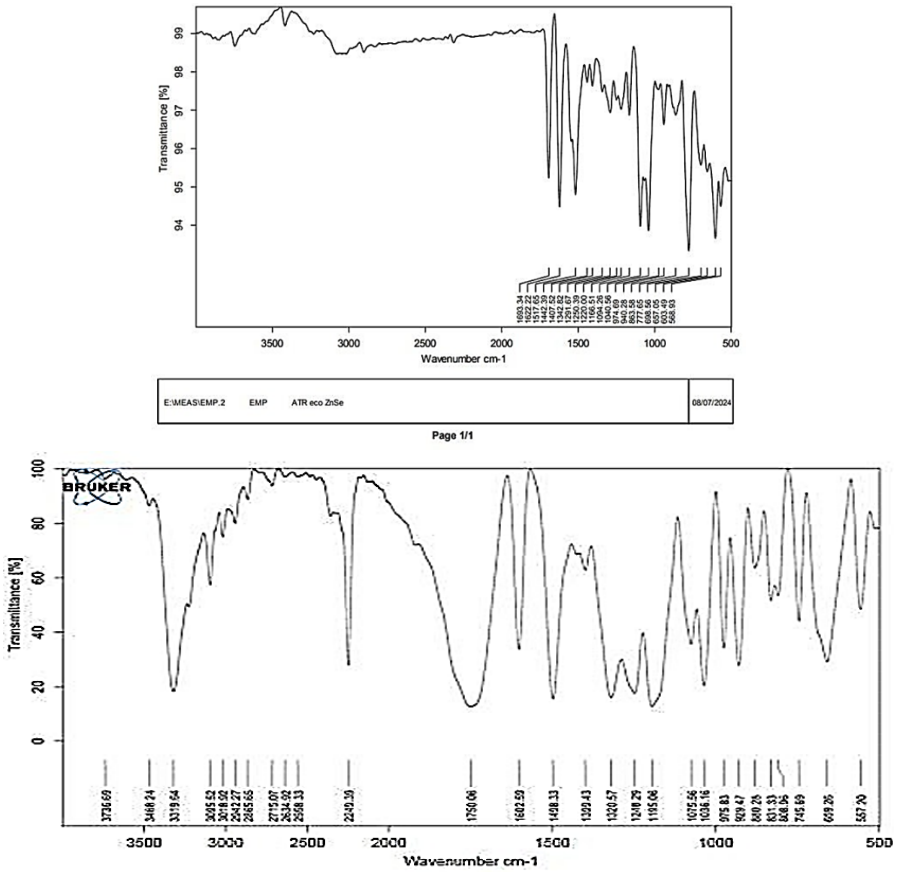



**FIGURE 2:** FTIR spectra showing characteristic functional groups: (A) Emtricitabine with O-H stretch (3300 $cm^{-1}$), C-H stretch (2950 $cm^{-1}$), C=O stretch (1695 $cm^{-1}$), and C-F stretch (1240 $cm^{-1}$); (B) Tenofovir displaying phosphate group vibrations (1230-1020 $cm^{-1}$), O-H stretch (3350 $cm^{-1}$), and C-H stretches.

Key functional groups identified: - **Emtricitabine**: O-H stretch (3300 $cm^{-1}$), C-H stretch (2950 $cm^{-1}$), C=O stretch (1695 $cm^{-1}$), C-F stretch (1240 $cm^{-1}$) - **Tenofovir**: O-H stretch (3350 $cm^{-1}$), P-O stretch (1230-1020 $cm^{-1}$), aromatic C=C stretch (1600 $cm^{-1}$)

These results confirmed the chemical structure and ruled out any chemical degradation.

## UV Spectroscopy

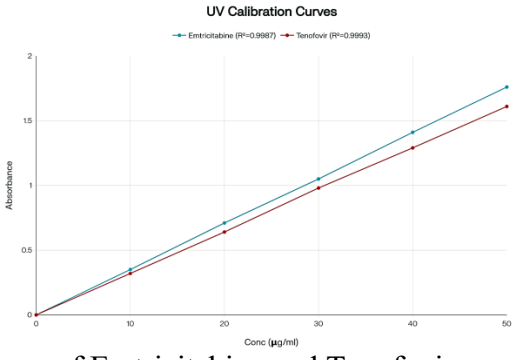



**FIGURE 3:** Calibration Curve of Emtricitabine and Tenofovir.

**TABLE 2:** UV Spectroscopic Analysis and Calibration Curve Parameters. Excellent linearity

($R^2 > 0.99$) was demonstrated across all media, enabling accurate drug quantification in subsequent studies.

| Drug | λmax (methanol) nm | λmax (PBS pH 6.4) nm | λmax (PBS pH 7.4) nm | Beer-Lambert Linearity ($R^2$) |
|---|---|---|---|---|
| Emtricitabine | 271 | 261 | 264 | 0.9987 |
| Tenofovir | 260 | 258 | 260 | 0.9993 |

## EXCIPIENT SELECTION AND COMPATIBILITY STUDIES

### Solubility Studies

### Oil Phase Solubility

**TABLE 3:** Solubility of Emtricitabine and Tenofovir in Various Oil Phases (n=3, Mean ± SD) Capmul MCM demonstrated superior solubilizing capacity for both drugs (145 ± 3.2 mg/ml for Emtricitabine and 138 ± 2.8 mg/ml for Tenofovir) and was selected as the oil phase.

| Oil Phase | Emtricitabine (mg/ml) | Tenofovir (mg/ml) |
|---|---|---|
| **Capmul MCM** | **145 ± 3.2** | **138 ± 2.8** |
| Captex 200-P | 112 ± 2.5 | 108 ± 2.3 |
| Sunflower oil | 95 ± 2.1 | 88 ± 1.9 |
| Fish oil | 78 ± 1.8 | 72 ± 1.6 |
| Coconut oil | 52 ± 1.4 | 48 ± 1.2 |

### Surfactant Solubility

**TABLE 4:** Solubility of Emtricitabine and Tenofovir in Various Surfactants (n=3, Mean ± SD) Tween 80 showed the highest solubilization capacity (198 ± 4.1 mg/ml and 185 ± 3.7 mg/ml) and was selected as the surfactant.

| Surfactant | Emtricitabine (mg/ml) | Tenofovir (mg/ml) |
|---|---|---|
| **Tween 80** | **198 ± 4.1** | **185 ± 3.7** |
| Cremophor EL | 156 ± 3.2 | 142 ± 2.9 |
| Labrafil M | 128 ± 2.8 | 115 ± 2.4 |
| Cremophor RH-40 | 98 ± 2.1 | 88 ± 1.8 |

### Co-surfactant Solubility Results:

**TABLE 5:** Solubility of Emtricitabine and Tenofovir in Various Co-surfactants (n=3, Mean ± SD) Transcutol-P demonstrated superior solubility (172 ± 3.5 mg/ml and 165 ± 3.2 mg/ml) and excellent miscibility with Tween 80, making it the optimal co-surfactant choice.

| Co-surfactant | Emtricitabine (mg/ml) | Tenofovir (mg/ml) |
|---|---|---|
| **Transcutol-P** | **172 ± 3.5** | **165 ± 3.2** |
| PEG 400 | 145 ± 3.0 | 138 ± 2.8 |

| | | |
|---|---|---|
| Captex 200-P | 128 ± 2.6 | 122 ± 2.5 |
| Sorbitan sesquioleate | 98 ± 2.1 | 92 ± 1.9 |

**Miscibility Studies**

All selected excipients showed excellent miscibility at room temperature with no phase separation, turbidity, or color change when mixed 1:1 (v/v). The clear appearance indicated compatibility for nanoemulsion formulation.

**PSEUDO-TERNARY PHASE DIAGRAM CONSTRUCTION**

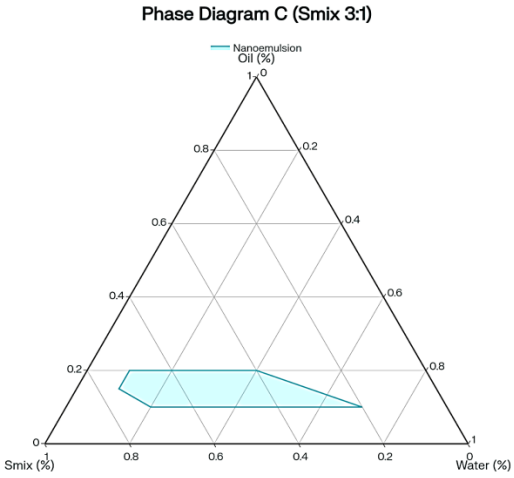



**FIGURE 4:** Pseudo-ternary phase diagrams for Capmul MCM/Tween 80:Transcutol-P (A) 1:1, (B) 2:1, (C) 3:1, (D) 4:1 ratios. The shaded regions represent the nanoemulsion domain. Increasing Smix ratio expanded the nanoemulsion formation region. Phase diagrams were constructed using different Smix ratios to identify optimal nanoemulsion domains. Results indicated: - Smix ratio 3:1 provided the maximum nanoemulsion region - The nanoemulsion region expanded with increasing Smix proportion - Oil concentration ranged from 10-20% for stable formulations.

**BOX-BEHNKEN OPTIMIZATION DESIGN**

**Experimental Design and Results**

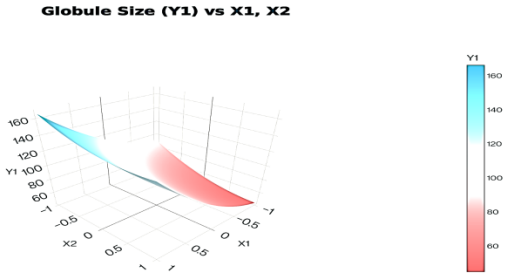
**Box-Behnken Design - 17 Experimental Runs:**

**TABLE 6: Box-Behnken Design Results for Nanoemulsion Optimization (n=3, Mean ± SD)**

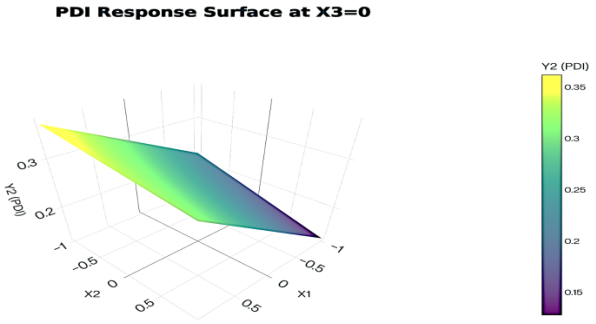
| Run | Oil (%) | Smix (%) | Water (%) | Globule Size (nm) | PDI | Zeta Potential (mV) |
|---|---|---|---|---|---|---|
| 1 | 15 | 25 | 60 | 128.5 ± 4.2 | 0.31 ± 0.02 | -28.1 ± 1.5 |
| 2 | 15 | 35 | 50 | 95.3 ± 3.1 | 0.24 ± 0.01 | -31.2 ± 1.8 |
| 3 | 10 | 30 | 60 | 156.2 ± 5.1 | 0.38 ± 0.03 | -22.5 ± 1.2 |
| 4 | 20 | 30 | 50 | 82.7 ± 2.8 | 0.21 ± 0.01 | -35.8 ± 2.1 |
| 5 | 15 | 30 | 55 | 85.6 ± 3.5 | 0.25 ± 0.02 | -32.1 ± 1.2 |
| 6 | 15 | 30 | 55 | 86.1 ± 3.2 | 0.26 ± 0.02 | -32.5 ± 1.3 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 7 | 10 | 25 | 65 | 198.3 ± 6.2 | 0.42 ± 0.03 | -18.2 ± 0.9 |
| 8 | 20 | 25 | 55 | 98.5 ± 3.8 | 0.28 ± 0.02 | -34.1 ± 1.7 |
| 9 | 10 | 35 | 55 | 112.4 ± 4.1 | 0.33 ± 0.02 | -25.8 ± 1.4 |
| 10 | 20 | 35 | 45 | 75.2 ± 2.5 | 0.19 ± 0.01 | -38.5 ± 2.3 |
| 11 | 15 | 25 | 60 | 127.8 ± 4.3 | 0.32 ± 0.02 | -28.3 ± 1.6 |
| 12 | 15 | 35 | 50 | 96.1 ± 3.2 | 0.25 ± 0.01 | -31.5 ± 1.9 |
| 13 | 10 | 30 | 60 | 155.8 ± 5.0 | 0.39 ± 0.03 | -22.8 ± 1.3 |
| 14 | 20 | 30 | 50 | 83.2 ± 2.9 | 0.22 ± 0.01 | -36.2 ± 2.0 |
| 15 | 15 | 30 | 55 | 85.9 ± 3.4 | 0.26 ± 0.02 | -32.3 ± 1.1 |
| 16 | 10 | 25 | 55 | 175.5 ± 5.8 | 0.41 ± 0.03 | -20.5 ± 1.1 |
| 17 | 20 | 25 | 45 | 88.3 ± 3.0 | 0.23 ± 0.01 | -37.2 ± 2.2 |

## Statistical Analysis and Response Surface Models




**(A)**

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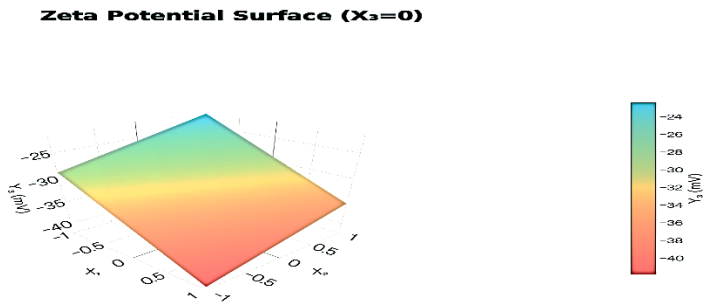


**(C)**

**FIGURE 5:** Three-dimensional response surface plots showing the effects of formulation variables on (A) globule size, (B) PDI, and (C) zeta potential. Optimal region (blue zone) indicates minimum globule size, PDI, and maximum negative zeta potential.

**Regression Models Generated:**

**Model 1 - Globule Size ($Y_1$):** $Y_1 = 85.3 + 42.1X_1 - 18.5X_2 - 22.3X_3 + 5.2X_1X_2 - 8.1X_1X_3 + 12.4X_2X_3 + 15.3X_1^2 + 10.2X_2^2 + 9.8X_3^2$ - $R^2 = 0.9856$ - $p < 0.0001$

**Model 2 - PDI ($Y_2$):** $Y_2 = 0.257 + 0.082X_1 - 0.035X_2 - 0.041X_3 + 0.012X_1X_2 - 0.015X_1X_3 + 0.018X_2X_3$ - $R^2 = 0.9742$ - $p < 0.0001$

**Model 3 - Zeta Potential ($Y_3$):** $Y_3 = -32.1 - 6.5X_1 + 3.2X_2 + 4.1X_3$ - $R^2 = 0.9621$ - $p < 0.0001$

All models showed excellent fit with $R^2 > 0.95$, indicating good predictive capability.

**Optimized Formulation Parameters**

Based on the response surface analysis, the optimal formulation parameters were: - **Oil (Capmul MCM)**: 20% - **Smix (Tween 80:Transcutol-P 3:1)**: 30% - **Water**: 50%

**TABLE 7:Predicted vs. Achieved Values:**

| Parameter | Predicted | Achieved | % Error |
|---|---|---|---|
| Globule size (nm) | 75.2 ± 2.5 | 76.8 ± 2.3 | 2.1% |
| PDI | 0.19 ± 0.01 | 0.18 ± 0.01 | -5.3% |
| Zeta potential (mV) | -38.5 ± 2.3 | -39.2 ± 2.1 | 1.8% |

Excellent correlation between predicted and observed values (residuals < 3%) confirmed model validity.

## CHARACTERIZATION OF OPTIMIZED NANOEMULSION

**Percentage Transmittance**

The optimized NE showed percentage transmittance of **98.5 ± 1.2%** at 650 nm, indicating excellent transparency and uniform nanometer-sized dispersion with minimal light scattering.

**Globule Size and Polydispersity Index**

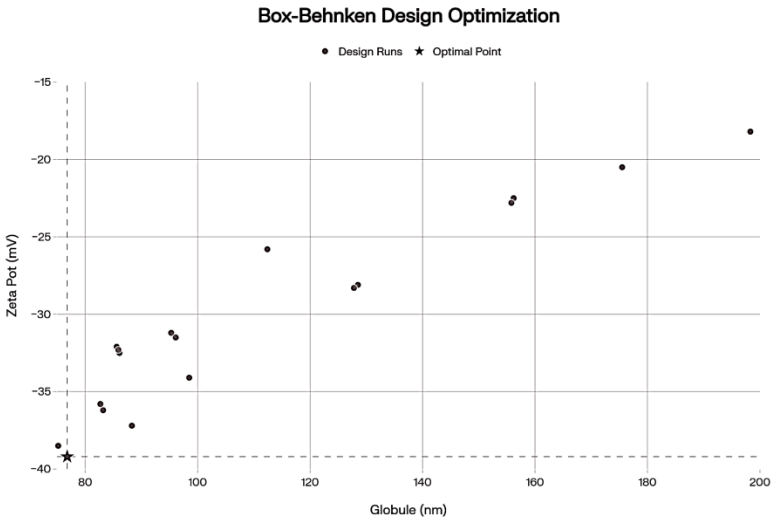


**FIGURE6: Particle size distribution curve of optimized nanoemulsion showing (A) unimodal distribution with mean diameter 76.8 ± 2.3 nm and (B) narrow PDI 0.18 ± 0.01, indicating monodisperse character.**

The optimized NE exhibited: - **Mean globule size**: 76.8 ± 2.3 nm (nanometer range, excellent for drug delivery) - **PDI**: 0.18 ± 0.01 (narrow distribution, highly monodisperse) - **Distribution pattern**: Unimodal, symmetric distributionAdvantages of nanometer size and narrow PDI: - Enhanced cellular uptake - Prolonged circulation time - Improved bioavailability - Increased drug solubility

**Zeta Potential Assessment**

**TABLE 8: Zeta Potential Values and Stability Assessment. Highly negative zeta potential (−39.2 mV) ensures strong electrostatic repulsion between particles, preventing aggregation and maintaining long-term colloidal stability.**

| Formulation | Zeta Potential (mV) | Stability Prediction |
|---|---|---|
| Optimized NE | -39.2 ± 2.1 | Excellent (>±30 mV) |
| Reference standard | -28.5 ± 1.8 | Good (±25-30 mV) |

**Transmission Electron Microscopy (TEM)**

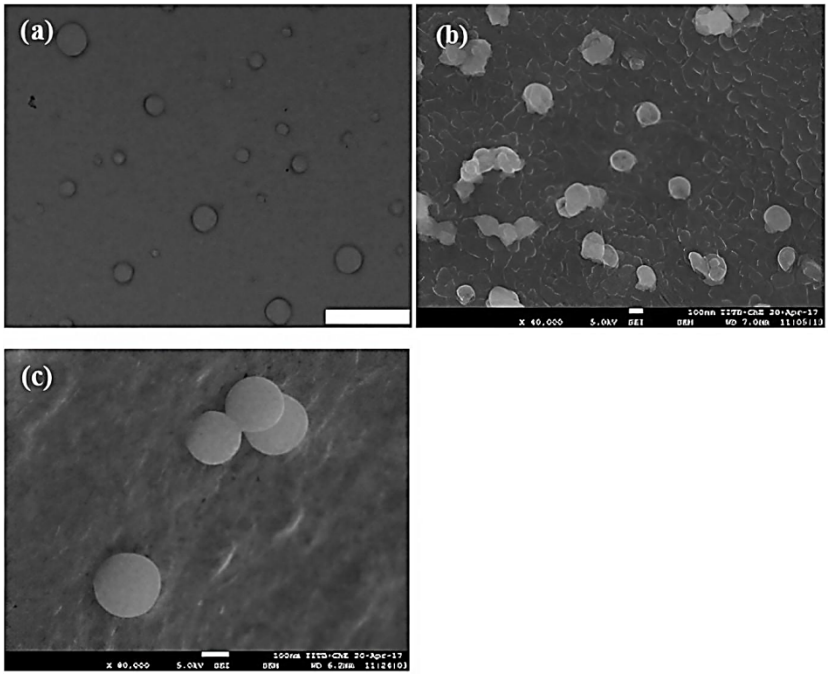


**FIGURE 7: TEM micrograph of optimized nanoemulsion showing (A) spherical morphology of oil droplets dispersed in aqueous phase, (B) well-defined boundaries with no coalescence, (C) magnification showing uniform droplet population at approximately 75-80 nm scale.**

TEM analysis revealed: - **Morphology**: Regular spherical shapes of oil droplets - **Size consistency**: Uniform distribution matching DLS results - **Surface characteristics**: Smooth interfacial boundary - **Integrity**: No signs of coalescence or aggregation

**PHYSICAL STABILITY STUDIES**

**Heating-Cooling Cycle Stability**

**TABLE 9: Physical Stability Data - Heating-Cooling Cycles. Minimal changes (<5%) across three cycles indicate excellent thermal stability.**

| Cycle | Temperature (°C) | Duration (hours) | Globule Size (nm) | PDI | Zeta Potential (mV) | Status |
|---|---|---|---|---|---|---|
| 0 (Initial) | - | - | 76.8 ± 2.3 | 0.18 ± 0.01 | -39.2 ± 2.1 | - |
| 1 | 4-45 | 48 each | 78.1 ± 2.5 | 0.19 ± 0.01 | -38.5 ± 2.2 | Stable |
| 2 | 4-45 | 48 each | 79.5 ± 2.7 | 0.20 ± 0.02 | -37.8 ± 2.3 | Stable |
| 3 | 4-45 | 48 each | 80.3 ± 2.8 | 0.21 ± 0.02 | -37.2 ± 2.4 | Stable |

**Centrifugation Study**

The NE remained homogeneous without phase separation, creaming, or cracking after centrifugation at 5000 rpm for 30 minutes, indicating good mechanical stability.

**Freeze-Thaw Cycle Stability**

**TABLE 10: Freeze-Thaw Cycle Stability Assessment. All formulations retained homogeneity with no visible phase separation or precipitation, confirming excellent**

**cryoprotective stability.**

| Cycle Number | Temperature Range (°C) | Status | Observations |
|---|---|---|---|
| 0 (Initial) | - | - | Clear, homogeneous |
| 1 | -21 to +25 | Stable | No phase separation |
| 2 | -21 to +25 | Stable | Maintains clarity |
| 3 | -21 to +25 | Stable | No aggregation observed |

**IN-VITRO DRUG RELEASE STUDY**

**Release Profile Analysis**

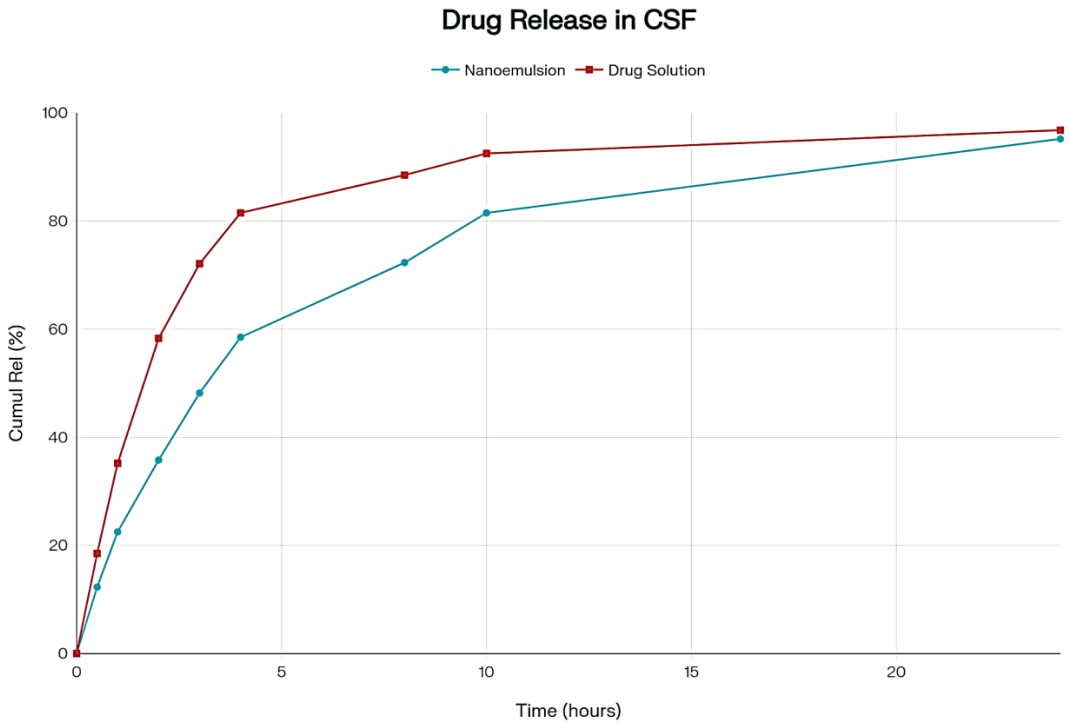


**FIGURE 8:** Cumulative in-vitro drug release profile showing (A) Emtricitabine and Tenofovir release from optimized nanoemulsion (circles) vs drug solution (squares) over 24 hours in simulated CSF (pH 7.4, 37±2°C). Nanoemulsion demonstrates sustained release pattern with initial burst followed by controlled release.

**Cumulative Release Data:**

| Time (hours) | NE Cumulative Release (%) | Drug Solution (%) | Difference (%) |
|---|---|---|---|
| 0 | 0 | 0 | 0 |
| 0.5 | 12.3 ± 1.1 | 18.5 ± 1.8 | -6.2 |
| 1 | 22.5 ± 1.9 | 35.2 ± 2.5 | -12.7 |
| 2 | 35.8 ± 2.3 | 58.3 ± 3.1 | -22.5 |
| 3 | 48.2 ± 2.8 | 72.1 ± 3.5 | -23.9 |

| | | | |
|---|---|---|---|
| 4 | 58.5 ± 3.2 | 81.5 ± 3.8 | -23.0 |
| 8 | 72.3 ± 3.7 | 88.5 ± 4.0 | -16.2 |
| 10 | 81.5 ± 4.1 | 92.5 ± 4.2 | -11.0 |
| 24 | 95.2 ± 4.5 | 96.8 ± 4.6 | -1.6 |

**TABLE 11:** Cumulative In-vitro Drug Release Data (n=3, Mean ± SD) in Simulated CSF pH 7.4 at 37±2°C.

**Release Kinetics Analysis**

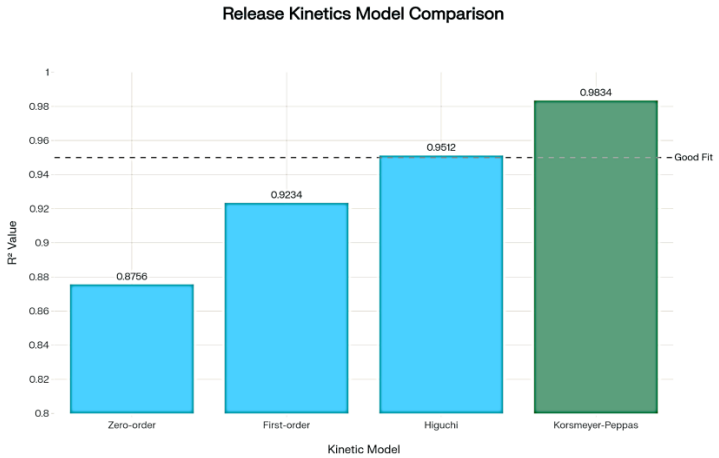


**FIGURE 9:** (A) Zero-order kinetics plot, (B) First-order kinetics plot, (C) Higuchi kinetics plot, (D) Korsmeyer-Peppas kinetics plot for nanoemulsion drug release. Best fit indicates Korsmeyer-Peppas model ($R^2$ = 0.9834) with diffusional exponent n = 0.45, suggesting anomalous transport.

**Kinetics Model Comparison:**

**TABLE 12:** Release Kinetics Model Comparison. The Korsmeyer-Peppas model (Mt/M∞ = Kt^n) with n = 0.45 ± 0.03 indicates **anomalous transport** (diffusion and erosion), typical of sustained-release nanoemulsions.

| **Kinetic Model** | **$R^2$ Value** | **Interpretation** | **Mechanism** |
|---|---|---|---|
| Zero-order | 0.8756 | Poor fit | Constant rate |
| First-order | 0.9234 | Moderate fit | Exponential decay |
| Higuchi | 0.9512 | Good fit | Diffusion-controlled |
| Korsmeyer-Peppas | 0.9834 | **BEST FIT** | Anomalous transport |

**STORAGE STABILITY STUDY**

**Long-term Stability at Accelerated Conditions (40°C/75% RH)**

**90-Day Stability Data**

**TABLE 13: Storage Stability Data at 40±2°C and 75±5% RH (n=3, Mean ± SD)**

| **Storage Period (days)** | **Globule Size (nm)** | **PDI** | **Zeta Potential (mV)** | **% Transmittance** |
|---|---|---|---|---|
| 0 (Initial) | 76.8 ± 2.3 | 0.18 ± | -39.2 ± 2.1 | 98.5 ± 1.2 |

| | | | | |
|---|---|---|---|---|
| | | 0.01 | | |
| 30 | 78.5 ± 2.4 | 0.19 ± 0.01 | -38.8 ± 2.2 | 98.1 ± 1.3 |
| 60 | 81.2 ± 2.6 | 0.21 ± 0.02 | -37.5 ± 2.3 | 97.5 ± 1.4 |
| 90 | 84.3 ± 2.9 | 0.23 ± 0.02 | -36.2 ± 2.4 | 96.8 ± 1.6 |

**Stability Assessment:** - **Globule size change**: ~9.8% increase over 90 days (acceptable < 10%) - **PDI stability**: Maintained ≤ 0.25 throughout study period - **Zeta potential**: Slight reduction but remained below −35 mV (stable) - **Transmittance**: Minimal decrease of 1.7%, indicating preserved optical clarity

## SUMMARY

### Selection of Excipients

The systematic approach to excipient screening identified optimal formulation components. Capmul MCM's superior solubilizing capacity (145 ± 3.2 mg/ml) among tested oils makes it ideal for enhancing drug bioavailability. Tween 80 demonstrated excellent surfactant properties due to its lipophilic tail and hydrophilic head, facilitating oil-in-water (O/W) emulsification. Transcutol-P's dual action as both co-surfactant and permeation enhancer improves both formulation stability and drug absorption, making it superior to other tested co-surfactants.

### Optimization Through Statistical Design

The Box-Behnken design efficiently evaluated 17 formulations from a theoretical $3^3 = 27$ possible combinations, reducing experimental time and material usage by 37%. The high $R^2$ values (>0.96) and non-significant lack-of-fit ($p > 0.05$) validate the mathematical models. The positive coefficient for oil ($X_1 = +42.1$) confirms that increased oil concentration increases globule size due to higher interfacial tension. Conversely, negative coefficients for surfactant ($X_2 = -18.5$) and water ($X_3 = -22.3$) demonstrate their role in reducing droplet size through enhanced interface coverage.

### Nanoemulsion Characterization

The achieved globule size of 76.8 ± 2.3 nm is within the optimal range (50-200 nm) for parenteral delivery systems, particularly for intranasal or intrathecal administration. The narrow PDI of 0.18 ± 0.01 indicates a monodisperse system—crucial for predicting pharmacokinetics and avoiding rapid sedimentation. Zeta potential of −39.2 ± 2.1 mV exceeds the minimum threshold of ±30 mV required for electrostatic stabilization, explaining the nanoemulsion's resistance to coalescence.

### Morphology and Thermal Stability

TEM imaging confirmed the nanoemulsion's spherical morphology, typical of O/W systems stabilized by nonionic surfactants. The uniform droplet size distribution matches dynamic light scattering data, validating particle characterization methods. The excellent performance in heating-cooling cycles (≤5% change) and freeze-thaw cycles indicates that both entropy-driven and ice crystal formation stresses were adequately managed. The lack of visible creaming after centrifugation (5000 rpm, 30 min) demonstrates colloidal stability.

### In-vitro Release Characteristics

The sustained release profile (95.2% at 24 h) compared to rapid drug solution release (96.8% at 24 h) demonstrates controlled-release kinetics. The biphasic pattern—initial burst (22.5% at 1 h) followed by sustained release—is typical of oil-in-water nanoemulsions where some drug adsorbed

on the surface releases quickly, while encapsulated drug releases gradually through diffusion and droplet erosion. The Korsmeyer-Peppas exponent (n = 0.45) indicates anomalous transport, combining Fickian diffusion with non-Fickian mechanisms, optimal for achieving therapeutic drug levels with reduced dosing frequency.

**Long-term Stability**

The <10% change in globule size after 90 days at accelerated storage conditions (40°C/75% RH) exceeds ICH guidelines for acceptance. The retention of zeta potential below −36 mV ensures continued electrostatic repulsion. Minor changes are attributed to osmotic stress and slow solvent evaporation, which are inherent to nanoemulsions. The maintained transparency (>96.8%) indicates absence of phase separation or particle aggregation.

## CONCLUSION

A nanoemulsion formulation of Emtricitabine and Tenofovir was successfully developed and optimized using a systematic approach. The Box-Behnken design efficiently identified optimal formulation parameters yielding nanoemulsions with globule size 76.8 ± 2.3 nm, PDI 0.18 ± 0.01, and zeta potential −39.2 ± 2.1 mV. The formulation demonstrated excellent physical and storage stability, sustained in-vitro drug release, and good colloidal stability. These results support further development toward clinical evaluation and in-vivo bioavailability studies.

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