Research Article

Development And In Vivo Evaluation Of An Intranasal Nano Emulsion-Based Nose-To-Brain Delivery System For Enhanced Therapeutic Efficacy In Alzheimer's Disease

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Abstract

Nanoemulsions, which are nanostructured and colloidally stabilized materials, have emerged as a promising approach for drug delivery. A major challenge in treating Alzheimer's disease (AD) is overcoming the blood-brain barrier (BBB) to deliver therapeutic agents effectively to the brain. The goal of this study was to develop a Rivastigmine (RSG) loaded nanoemulsion that can enhance the treatment for AD patients. We formulated a nanoemulsion containing RSG by incorporating pyridoxine, an essential vitamin for central nervous system function, along with linseed oil, which acted as the lipophilic phase in the formulation. Nine different formulations were created using the emulsification method and were then characterized. The formulation labeled "RF6" exhibited the highest drug entrapment efficiency, with 83.25% of the drug retained, likely due to its higher linseed oil content. In-vitro drug release studies conducted using a Franz diffusion cell showed that the "RF6" formulation provided a sustained release of the drug. The optimal formulation, with a globular size of 203.4 nm, was further analyzed using various techniques, including zeta potential analysis, ATR, DSC, and XRD. Cognitive performance in Long-Evans rats with AD was evaluated using the Morris Water Maze (MWM) model. The findings of this study suggest that the combination of RSG nanoemulsion with linseed oil and pyridoxine reduced the travel distance in the animal model, offering potential therapeutic benefits for AD patients.

Keywords: Rivastigmine, Nose to Brain, Nano-emulsion.

Introduction

Alzheimer's disease (AD) is a prevalent neurodegenerative disorder that primarily affects memory and cognitive functions. The underlying causes of AD are multifactorial, involving factors such as disruptions in cholinergic signaling, oxidative stress, glutamate toxicity, and the buildup of amyloid β plaques. While current treatments only address the symptoms, there are no medications available that provide a definitive cure for the disease.

Rivastigmine (RSG) is classified as a cholinesterase inhibitor, a pharmacological group that reduces the activity of both acetyl cholinesterase and butyrylcholinesterase enzymes. This medication is primarily used to manage dementia symptoms, such as memory loss and cognitive decline, typically seen in patients with Alzheimer's disease (AD) or Parkinson's disease. Although RSG can alleviate some cognitive impairments, it does not cure or modify the progression of AD or Parkinson's disease. However, it has shown potential to improve cognitive function in certain individuals. One of its advantages is its limited interaction with other medications, making it suitable for elderly patients who may be on multiple drugs [1-4].

A challenge with RSG, however, lies in its significant hydrophilicity, which limits its ability to cross the blood-brain barrier (BBB), thus requiring frequent dosing and potentially leading to cholinergic side effects. To overcome this, various strategies have been proposed, such as enhancing the drug's lipophilicity, disrupting BBB intercellular junctions, and exploring alternative delivery methods like intranasal or parenteral routes. Furthermore, developing nanoscale formulations, nanoemulsions with a suitable lipophilic phase, and adding emulsifiers could improve brain penetration. Although intranasal administration could be an effective way to bypass the BBB, issues with nasal physiology, such as mucosal barriers and enzymatic activity, may affect its absorption and bioavailability. Currently, the only available formulations of RSG on the market for Alzheimer's treatment are the oral capsule (Exelon®) and the transdermal patch (Exelon® patch) [5-9].

Nanoemulsions are considered ideal carriers for delivering poorly water-soluble drugs, as they help enhance bioavailability. These systems are typically composed of oil, water, and a combination of surfactants and cosurfactants. The presence of

oil and surfactants provides an excellent solubilizing effect for hydrophobic drugs. By dispersing the drug-loaded oil into the water phase with the help of surfactants and cosurfactants, nanoemulsions form small droplets with nanometer-sized particles. This process improves the solubility and bioavailability of poorly soluble drugs. Recent research also indicates that naringenin-loaded nanoemulsions have demonstrated neuroprotective effects in Alzheimer's and Parkinson's disease models, highlighting the potential of nanoemulsions in developing therapies for neurodegenerative conditions [10-13].
The oral delivery of drugs to the brain can be hindered by the blood-brain barrier (BBB), often leading to low bioavailability. As a result, nasal administration has emerged as an alternative route, as it allows for direct brain-targeted drug delivery. Research has also shown that nasal transport of nanocomposites is a viable strategy for brain targeting. For instance, the use of drug-loaded nanoemulsions delivered through the nasal route could enhance the drug's brain delivery and bioavailability. Bhattamisra et al. found that intranasal delivery of rotigotine-loaded chitosan nanoparticles improved both brain targeting efficiency and bioavailability in rats. Other studies demonstrated that nanoemulsions facilitated the brain delivery of donepezil and memantine, two FDA-approved drugs for Alzheimer's disease, when administered intranasally. These findings highlight the potential of nanotechnology to improve nasal drug delivery for treating neurological conditions like Alzheimer's disease.

Material and Method

Materials

The RSG sample was obtained as a gift from Sun Pharmaceutical Pvt Ltd, located in Ahmedabad, India. Tween 80 was sourced from Loba Chemie in Mumbai, India. Various oils, including linseed oil (Avisa oil, Vijayawada), olive oil (Borges, Spain), almond oil (Roghan Badam, India), castor oil (Wow, Mumbai), coconut oil (Nuerma Science, Mumbai), sunflower oil (Deve Herbes, India), and peanut oil (Appu Edible, Ahmedabad), were purchased from a local drug store in Vijayawada, Andhra Pradesh. The manufacturers of these oils have claimed that they are 100% pure. All materials used in the study were of high analytical grade.

Solubility analysis

The selection of the oil was based on a detailed evaluation of its solubility properties. An analysis of RSG solubility in various oils was carried out according to the procedure outlined by Ashfaq M et al., with minor adjustments. To determine the solubility of RSG in different oils, a sufficient quantity of RSG was added to 5 mL samples of each oil, including olive oil, almond oil, castor oil, coconut oil, sunflower oil, linseed oil, and peanut oil, all placed in 10 mL stoppered vials. These mixtures were then subjected to agitation using a vortex mixer (Neuation Vortex Mixer- 4200, Mumbai, India). Afterward, the vials were placed on an isothermal shaker set at a constant temperature of 25 ± 2ºC (Orbital Shaker, Labline, India) for 72 hours to reach equilibrium. This procedure, known as the shake flask method, was followed. Upon reaching equilibrium, the samples were removed from the shaker and centrifuged at 10,000 rpm for one hour using a high-speed centrifuge (High centrifuger, Thermo Scientific, Germany). The liquid portion of each mixture was separated, dissolved in methanol, and filtered through a 0.22µm membrane filter. The drug concentration was determined using a UV spectrophotometer (Shimadzu, UV2600i, Japan). Prior to the solubility tests, the UV spectroscopy method was developed and validated for accuracy. To create the primary stock solution, the required amount of RSG was dissolved in 50 mL of methanol. The final volume was then adjusted to 100 mL by adding the appropriate oil. Stock solutions were prepared for each oil mentioned above, and the solutions were bath-sonicated for about 15 minutes to ensure clarity. Dilutions (5µg/mL) were made using the respective oils, and their absorbance maxima were determined using the UV spectrophotometer (UV spectrum was not included in the manuscript). The method was also validated for parameters such as linearity, accuracy, precision, and ruggedness (data available in the supplementary file). The solubility experiments were conducted in triplicate, and the results were expressed as the mean ± standard deviation.

Preparation of RSG loaded nanoemulsion

The RSG-loaded nanoemulsion was prepared using the titration method as described by Tayeb et al. (2021). In this process, the solubility of a specific amount of RSG was achieved by blending it with the oil phase, specifically Linseed oil, using a vortex mixer (Neuation Vortex Mixer-4200, Mumbai, India). A predetermined quantity of surfactant (Tween 80) was then added to the mixture, which was continuously stirred with a magnetic stirrer (Remi 5MLH, Mumbai, India). Distilled water was gradually incorporated into the oil phase drop by drop, while stirring was maintained. To ensure a transparent and

homogeneous nanoemulsion, the dispersion was sonicated for 10 minutes at an amplitude of 6% using a probe sonicator (UAI-PS20khz-900W, Ultra Autosonic, India).

Table 1: Formulation composition of RSG loaded nanoemulsion

Sr.no.	Ingredients	F1	F2	F3	F4	F5	F6	F7	F8	F9
1	RSG (mg)	3.7	3.7	3.7	3.7	3.7	3.7	3.7	3.7	3.7
2	Tween-80 (mL)	1	1	1	1	1	1	1	1	1
3	Pyridoxine (mg)	4	8	12	16	20	16	12	8	4
4	Linseed oil (mL)	1	2	3	4	5	6	5	4	3
5	Distill water (mL)	50	50	50	50	50	50	50	50	50

Characterization Of Nanoemulsion

The entrapment efficiency (EE) and drug loading (DL)

Roy et al. (2023) aimed to assess the effectiveness and methodology of their investigation. The process involved the use of a high-speed centrifugal filter (Remi R-8C, Hyderabad, India), which was operated for 10 minutes with 5 mL of nanoemulsion. After centrifugation, the supernatant was analyzed using a double-beam UV-Visible spectrophotometer (Shimadzu, UV 3600i, Japan) at 221 nm to determine the concentration of unbound RSG. Additionally, the total amount of RSG in the sample was quantified by employing a bath sonicator (Ultrasonicator, Verilux, Hong Kong), where the nanoemulsion was sonicated in an ethanol-water mixture. The encapsulation efficiency (EE) and drug loading (DL) were calculated as percentages using the formulas presented in Equation 1 and Equation 2.

$$EE\ (\%) = \frac{\text{Total weight of RSG in nanoemulsion} - \text{Weight of free RSG in nanoemulsion}}{\text{Total weight of RSG in nanoemulsion}} \times 100 \quad \text{(Equation 1)}$$

$$DL\ (\%) = \frac{\text{Drug content in nanoemulsion}}{\text{Total weight of nanoemulsion}} \times 100 \quad \text{(Equation 2)}$$

Viscosity of nanoemulsion

The viscosity of the nanoemulsions was measured using a Brookfield viscometer (model DV-II+ Pro, Chennai, India) fitted with spindle number 64. Viscosity and torque values were recorded at rotational speeds of 50 and 100 rpm over a 30-second period. This method was consistently applied across all formulations. The reported viscosity was calculated as the average value from the measurements taken at the specified rotational speeds.

pH determination

The pH of the prepared nanoemulsions was determined using a digital pH meter. A 100 mL volumetric flask was used to hold 50 mL of the formulation, which was accurately measured and weighed. The pH was assessed by inserting the pH electrode (Eutech, Mumbai, India) into the formulation. The readings were taken three times, and the average value was calculated.

Temperature dependent stability by heating-cooling cycle

The experiment was carried out to assess the stability of the nanoemulsion in response to temperature variations. Ali et al. followed the established methodology for the study. Over a 48-hour period, the vials were subjected to temperature fluctuations ranging from 4°C to 40°C. The process was repeated cyclically six times to monitor and document the stability of the nanoemulsion.

Centrifugation

The formulations developed were subjected to centrifugation using a Remi R-8C centrifuge (India) as part of the centrifugation study. The method, originally proposed by Bayanati M et al. in 2021, was adapted for this research. The centrifugation was carried out at a speed of 5,000 revolutions per minute for 30 minutes, during which the phase separation was observed visually.

Freeze thaw method

The technique was chosen based on the successful research by Liu C et al., which demonstrated the thermodynamic stability of the nanoemulsion. In accordance with the previously described method, the samples were stored in a freezer (Haier, Mumbai) at -20°C for 20 hours, followed by exposure to 35°C. This process was repeated three times. The absence of cracking and phase separation in the nanoemulsion confirms its thermal stability and overall stability.

Release study of RSG by in-vitro performance

To assess the release of RSG from the developed formulations, an in-vitro diffusion method was applied using a Franz diffusion cell, following the procedure described by Vijaya Rani and colleagues. Before use, the cellophane membrane (with a pore size of 0.45 µm) was cut into uniform pieces measuring 6 cm x 2.5 cm. These segments were then immersed in sterilized distilled water for 12 hours. The drug release experiments were conducted with 15 mL of phosphate buffer (pH 6.8) at 37°C. A magnetic stirrer (HLS 200, Borosil, India) with continuous heating was used to maintain the temperature. A 2 mL sample of nanoemulsion was placed in the donor compartment, and 1 mL samples were withdrawn at specified time intervals, replacing the withdrawn volume with an equal amount of freshly prepared buffer to maintain the sink condition. If needed, the aliquots were diluted using the newly prepared buffer. Drug diffusion across the membrane was measured using a UV spectrophotometer at 221 nm (λmax). The phosphate buffer (pH 6.8) served as the blank in the experimental setup. The percentage of drug released over time is shown in Figure 2.

Globular size observation and zeta potential determination

The scanning electron microscope (SEM) is a widely used tool for analyzing nanometer-sized dosage forms, enabling the examination of their distribution and morphology. In this study, the SEM (HITACHI S-3700, Japan) was set with a working distance of 15,000 microns and an accelerating voltage of 14,500 volts, as indicated in a report by Roy H et al., 2023. The globular size was measured using the SZ-100V2 instrument by Horiba Scientific, India, which employs laser light scattering technology to determine the size of nanoemulsions and calculate their polydispersity index. This method works by measuring laser scattering intensity at varying angles, based on the size of particles and droplets in the dispersion. Larger globules scatter light at smaller angles, while smaller ones scatter light at larger angles. The zeta potential, measured with a Malvern instrument from the United Kingdom, indicates the electrical potential difference between the nanoemulsion and its dispersion medium. The samples are placed in a capillary cell equipped with two electrodes, across which a voltage is applied. Particles with a higher zeta potential move more rapidly through the medium, while those with lower zeta potential move slower. The zeta potential is then calculated using the electrophoretic mobility, following a specific formula. The data were collected and documented accordingly.

Attenuated total reflectance (ATR) study

The main aim of the ATR analysis was to explore how RSG (a specific compound) interacts and aligns with various excipients. The study was conducted using an ATR instrument (Bruker OPUS; Version 7.5, Germany). The RSG, its formulated components, and the enhanced nanoemulsion were analyzed spectrally with a resolution of 1.0 cm-1, spanning a wave number range of 4000 to 600 cm-1. After positioning the samples accurately on the instrument, spectral data was collected to evaluate the necessary information.

Differential scanning colorimetry (DSC) observation

The impact of temperature changes on the behavior and interactions of drugs and excipients can be explored to predict how they might interact in a specific environment. Differential Scanning Calorimetry (DSC) is a thermoanalytical technique that monitors the appearance and disappearance of peaks corresponding to endothermic and exothermic reactions. By analyzing the position of these peaks, it is possible to infer the differences in heat absorption or release as the temperature increases. In this study, DSC measurements were carried out using the DSC 60A model (Shimadzu Corporation, Japan). Nitrogen gas was used to purge the system at a flow rate of 50 mL/min, and the resulting thermograms were analyzed for further insights.

X-ray diffraction (XRD) observation

X-ray Diffraction (XRD) analysis is a valuable technique for the thorough characterization of crystalline materials, enabling the identification of their chemical composition as well as the evaluation of crystallographic quality, particularly in cases

where preferred orientation exists. The experiment utilized an X-Ray Diffractometer (Shimadzu, XRD-7000, Japan), operating with Cu-Kα radiation at 40kV and 30mA. The X-ray diffraction data was collected at a scanning rate of 4° per minute, with a 2θ range from 10° to 40°. For upcoming research, an analysis was carried out to accurately determine the position, characteristics, and intensity of the peaks related to RSG and excipients, following the method described by Roy H et al. (2020).

In-vivo performance by Morris water maze (MWM) model

The Morris Water Maze (MWM) model is widely used in scientific studies to evaluate the cognitive function of rodents, particularly in the context of neurodegenerative diseases such as Alzheimer's Disease (AD). The search procedure followed the methodology outlined by Morris and later adapted by Petrásek et al. The study adhered to the ethical guidelines set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), and received approval under reference number 046/IAEC/NCPA/22-23. The study involved Long-Evans rats, each weighing between 200 and 250 grams, obtained from a local supplier, Vijaya Supplier, in Vijayawada. In the initial five days of the MWM trials, the rats were acclimatized to minimize stress.

The rats were randomly assigned to one of four groups, each consisting of six rats, as detailed in Table 2. Group I served as the control group, consisting of healthy rats maintained on a regular diet. Group II, as described in the study by Bhuvanendran et al., was treated with scopolamine (SP) at a dose of 1 mg/kg via intraperitoneal (IP) injection for 9 days, to induce a brain condition resembling AD. Group III, the positive control group, received both scopolamine (1 mg/kg) and rosiglitazone (RSG) (0.5 mg/kg) through IP injection. Group IV was treated with 1 mg/kg of the selected nanoemulsion formulation, alongside 1 mg/kg of scopolamine, delivered by IP injection. Lastly, Group V received 1.5 mg/kg of the optimal formulation via IP injection, with 1 mg/kg of scopolamine administered in the same manner.

The MWM trials were conducted in a circular pool, measuring 80 cm in diameter and 40 cm in depth, with water maintained at a temperature of 26°C±1°C. A platform with a diameter of 10 cm was positioned 1 cm above the water's surface. Following the protocol outlined by Stuchlik et al., the pool was divided into four equal sections, and a digital camera was used to record the movement of the rats as they navigated the pool.

Table 2: Groups and treatment while performing MWM model study

Groups	Treatment
Group-I	Control group (regular diet)
Group-II	scopolamine (1mg/kg)
Group-III	scopolamine (1mg/kg) + RSG (0.5 mg/kg)
Group-IV	scopolamine (1mg/kg) + optimal formulation (1mg/kg)
Group-V	scopolamine (1mg/kg) + optimal formulation (1.5 mg/kg)

Result and Discussion

Solubility study interpretation

The solubility of the drug in the oil phase plays a crucial role in the effectiveness of nanoemulsions by ensuring the drug remains dissolved. UV spectrophotometric analysis revealed absorbance maxima of 222 nm and 223 nm in olive and castor oil, respectively. For almond oil, the maxima were noted at 223 nm, and 221 nm for coconut oil. In sunflower and peanut oils, the observed maxima were 222 nm and 223 nm, respectively. Linseed oil showed an absorbance maximum at 223 nm. The calibration curve, established over a range of 2 µg/mL to 20 µg/mL, displayed a strong linear correlation across all oils. Accuracy studies involved the addition of a known quantity of the drug, with recovery studies indicating a recovery percentage greater than 99%, confirming the method's validity. Precision, both intra- and inter-day, was found to have a standard deviation of less than 2%. Ruggedness tests, carried out by two analysts for a 16 µg/mL concentration of RSG, showed no significant deviation, with the recovery percentages also indicating reliability of the UV spectroscopy method. Based on these results, the solubility profile of RSG was evaluated in the oils tested, with the results shown in Figure 1. Among the oils studied, RSG exhibited the highest solubility in linseed oil, at 25.89 ± 5.6 mg/mL, followed by coconut oil with a solubility of 19.16 ± 2.2 mg/mL. These findings suggest the most suitable oil phases for subsequent experiments.

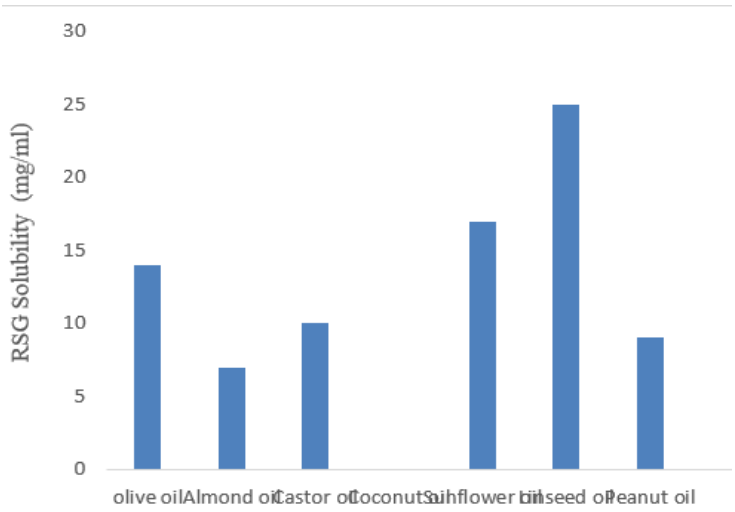


Figure 1: Before conducting the solubility study, a UV spectroscopy method was developed and validated to ensure accurate predictions. The solubility of RSG was highest in linseed oil, recorded at 24.88 ± 4.5 mg/mL, followed by coconut oil, which showed a solubility of 18.15 ± 2.1 mg/mL. The solubility experiments were performed in triplicate, and the results are presented as the mean ± standard deviation.

Characterization assessment of nanoemulsion

The stability of the nanoemulsions was assessed using a thermal cycling process that involved alternating heating and cooling. The nanoemulsions were maintained at the specified temperature for the required cycle duration. No phase separation was observed in any of the samples. Additionally, the formulations were subjected to centrifugation, as described in the methods section. All nanoemulsions, except for "RF6" and "RF7," showed stability, with phase separation occurring in the latter two. After a comprehensive review of the stability results, it was concluded that a higher concentration of linseed oil may hinder the formation of a stable emulsion, leading to phase separation. Previous research has explored this phase separation phenomenon, particularly focusing on the effect of the oil phase quantity. Phase separation was only observed in the "RF6" sample when using the "Freeze-thaw" method. This study aims to identify the optimal amount of linseed oil required to maintain stability during the Freeze-thaw process. Furthermore, the findings confirm the thermal stability of the nanoemulsions across a wide temperature range, from -20°C to 35°C, as detailed in Table 3.

Table 3: Assessment and evaluation of developed nanoemulsion

Formulations	The cyclic process of Heating cooling	Centrifugation	Freeze thaw method	Viscosity (cP)	pH	DL (%)	EE (%)
RF1	No phase separation	No phase separation	No-cracking	6.4±0.3	6.2±0.3	42.99±4.3	57.59±2.8
RF2	No phase separation	No phase separation	No-cracking	7.7±0.5	6.8±0.2	46.56±3.7	59.92±4.8
RF3	No phase separation	No phase separation	No-cracking	7.9±0.7	5.9±0.4	47.04±5.2	60.25±3.6

RF4	No phase separation	No phase separation	No-cracking	9.8±0.6	5.7±0.5	51.50±2.8	62.09±4.2
RF5	No phase separation	No phase separation	No-cracking	10.2±0.4	6.3±0.3	62.95±4.7	71.12±3.8
RF6	No phase separation	Phase separation	Cracking	13.5±0.8	6.9±0.4	72.36±3.9	83.25±4.9
RF7	No phase separation	Phase separation	No-cracking	11.8±0.7	6.5±0.3	69.82±4.3	76.65±3.3
RF8	No phase separation	No phase separation	No-cracking	9.4±0.3	6.8±0.6	57.25±3.2	65.39±5.2
RF9	No phase separation	No phase separation	No-cracking	7.2±0.5	6.2±0.4	50.26±5.2	60.89±3.2

No notable changes were observed in the pH during the pH testing conducted. The produced nanoemulsions displayed slightly acidic properties, with pH values ranging from 5.9 to 6.9. The viscosity of the formulations varied between 6.4 and 13.5 cP. A concentration-dependent relationship was noted between viscosity and linseed oil content. Formulations with higher oil phase proportions exhibited increased viscosity, whereas those with a lower oil phase showed a reduction in viscosity. The sample labeled "RF6" had the highest viscosity of 13.5 cP, while "RF1," which contained the least oil phase, had the lowest viscosity at 6.4 cP.

The drug loading (DL) in a pharmaceutical formulation, particularly within colloidal dispersion systems, can vary considerably, influenced by factors such as the polarity of the vehicle, polymer choice, the amount used, and interaction effects. In this study, DL values ranged from 42.99% to 72.36%, indicating an acceptable range. Similarly, the encapsulation efficiency (EE) was measured using the appropriate method previously detailed, with values varying from 57.59% to 83.25%. Among the formulations, "RF1" had the lowest drug concentration, while "RF6" exhibited the highest EE. The research showed that increasing the linseed oil content led to improved EE. A comparison of two samples, "RF5" and "RF7," which contained the same amount of linseed oil, revealed that "RF5" had a lower EE of 71.12%, while "RF7" demonstrated a higher EE of 76.65%. Additionally, "RF5," which had a significant concentration of pyridoxine and water solubility, suggested the possibility of solubilizing RSG into the external medium. This finding aligns with previous studies, which have reported a decrease in EE due to the hydrophilic nature of excipients.

Drug release and study from In-vitro study

Understanding the impact of drug-excipient combinations is vital for drug release studies. The methodology outlined by Ahmed MM et al. (2021) was followed in this investigation. In this setup, a cellulose acetate membrane was placed between the donor and receptor compartments to act as a barrier for diffusion. The collected data were analyzed and subsequently reported. All formulations showed an initial 10% release within the first 30 minutes of diffusion testing. According to the data presented in Figure 2, the formulation "RF6" demonstrated the slowest drug release, with a value of 70.62% after 12 hours. In contrast, the "RF1" formulation exhibited the fastest release, with 98.08% of the drug released within 8 hours during the dissolution test. The study found that the release of RSG from nanoemulsions was influenced by the amount of linseed oil used. Formulations with higher oil content showed slower release rates, while those with lower oil levels, like "RF1", released the drug more quickly. This could be due to the formation of a hydrophobic layer around RSG, created by the linseed oil. Additionally, pyridoxine significantly impacted the release profile, with "RF5" showing a higher release of 80.63% compared to "RF7", which released 78.40% of the drug. However, similar trends were not observed in other formulations where linseed oil was present in amounts less than 5 mL. This suggests that a stoichiometric interaction may occur between linseed oil and pyridoxine. Furthermore, the release kinetics and mechanisms of the drug were evaluated. Kinetics were studied by plotting zero-order and first-order release profiles, and the exact release mechanism was tested using the Higuchi, Hixson-Crowell, and Korsmeyer-Peppas models. The results revealed that the "R2" values for all formulations were higher for the zero-order model compared to the first-order, indicating a consistent release of RSG over time from all nanoemulsions. The release mechanism was further clarified, with the Higuchi model showing the highest "R2" values, ranging from 0.975 to 0.996 (data available in the supplementary file). The Higuchi model suggests that the drug is uniformly distributed throughout the matrix system. The study concluded that the release of RSG from the

nanoemulsion followed a diffusion-controlled mechanism. During the experiment, the sink conditions were carefully maintained, creating a concentration gradient that facilitated the diffusion of RSG.

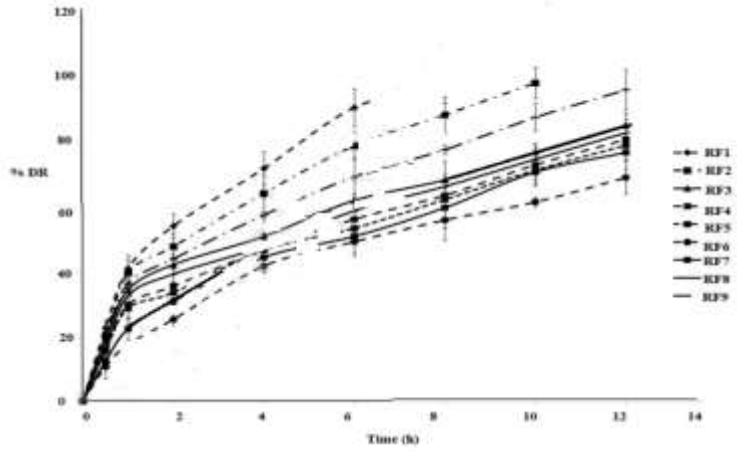
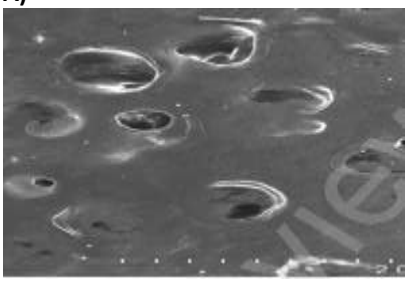


Figure 2: In the drug release study conducted using a Franz diffusion cell, all formulations demonstrated an initial 10% drug release within the first 30 minutes of the test. Among the formulations, "RF1" showed the highest drug release rate, achieving 98.08% within 8 hours of the dissolution test. On the other hand, "RF6" exhibited the slowest release, with only 70.62% of the drug released over a 12-hour period. The findings indicate that the release of RSG from the nanoemulsions is influenced by the amount of linseed oil used. Nanoemulsion formulations containing higher concentrations of oil showed a delayed release of RSG.

SEM and globule characterization

The SEM images presented in this study reveal a dispersed arrangement of the oil phase, which appears as small globules spread throughout the dispersion medium in the optimal nanoemulsion. The dispersed phase displayed considerable symmetry, as shown in Figure 3A. A globular size analysis was carried out on the best formulation (RF6), resulting in a mean globule diameter of 203.4 nm (Figure 3B). The polydispersity index (PDI) for this formulation was 0.480, indicating a slightly heterogeneous distribution of the globules, in line with previous findings by Danaei M et al., 2018. The zeta potential is commonly used to assess the dispersion and stability of nanodrug formulations and colloidal systems. A zeta potential value exceeding ±10 mV is generally considered indicative of a stable and long-lasting nanoemulsion. In contrast, a lower zeta potential suggests a tendency toward aggregation and flocculation. The formulation studied here exhibited a zeta potential of -16.7 mV, which suggests that it is a stable formulation.

A)



B)

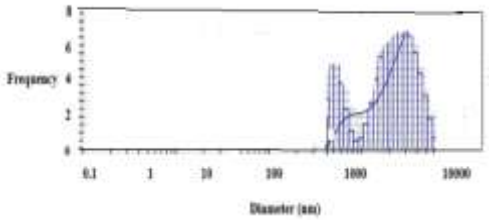


Figure 3: A SEM (Scanning Electron Microscopy) analysis was conducted to examine the microscopical properties of the selected nanoemulsion (A). The dispersed phase exhibited a high degree of symmetry. In addition, the globular size of RF6 was assessed (B), which revealed an average diameter of 203.4 nm.

ATR data interpretation

RSG is an ester synthesized via a condensation reaction involving carbamic acid and dimethyl amino ethyl phenol. The ATR spectra of RSG displayed a strong peak at 3516.83 cm^{-1}, which can be attributed to the moderate intensity from the stretching of the N-H bond within the amine functional group. A clear C=O stretching peak was observed at 1793.71 cm^{-1}. Typically, the C-N stretching in amine compounds appears as a moderate intensity peak within the 1250–1020 cm^{-1} range, and a medium peak was seen at 1229.46 cm^{-1}, corresponding to the C-N bond stretching. Additionally, another medium peak at 1655.44 cm^{-1} was assigned to the C=C bond stretching. The C-H stretching in the alkane group showed a moderate intensity peak at 1404.68 cm^{-1}, with another prominent C-H bending peak observed at 675.55 cm^{-1}, as shown in Figure 4A. Linseed oil, a lipid composed mainly of triglycerides, has a significant presence of α-linolenic acid. A notable C=O stretching peak was recorded at 1744.29 cm^{-1}, along with a broad, intense peak for the C-O bond at 1161.56 cm^{-1}. The C-H stretching exhibited a significant peak at 2923.88 cm^{-1}, with an additional peak at 1460.57 cm^{-1}, reflecting the scissoring motion of long-chain C-H bonds (Figure 4B). A peak at 721.48 cm^{-1} was linked to the rocking motion of long-chain methyl groups.

Pyridoxine, characterized by a pyridine nucleus attached to two hydroxyl methyl groups and one free hydroxyl group, showed an intense peak at 3228.40 cm^{-1} corresponding to the stretching of the hydroxyl (-OH) groups. Another distinct peak at 1383.20 cm^{-1} was associated with the bending of the hydroxyl group. The peaks at 1480.52 cm^{-1} and 1274.31 cm^{-1} corresponded to the stretching vibrations of the C=N and C-N bonds, respectively. Additionally, a peak at 744.45 cm^{-1} was observed, attributed to the rocking motion of long-chain methyl groups, as shown in Figure 4C.

The optimal formulation (RF6) was examined using ATR, and potential interactions were carefully analyzed, as illustrated in Figure 4D. A prominent peak at 3304.05 cm^{-1} indicated the NH stretching of the amine group in RSG. This peak was significantly more intense with a slight deviation when compared to the pure RSG compound. This interaction might result from the reaction between the amine and a free hydroxyl group in pyridoxine. A weaker peak at 2924.60 cm^{-1} corresponded to the free -OH stretching of pyridoxine. Additionally, a clear C=O stretching peak appeared at 1638.04 cm^{-1}, and a low-intensity peak at 1160.43 cm^{-1} was linked to the stretching of the carbon-carbon double bond (C=C) within the cyclic ring.

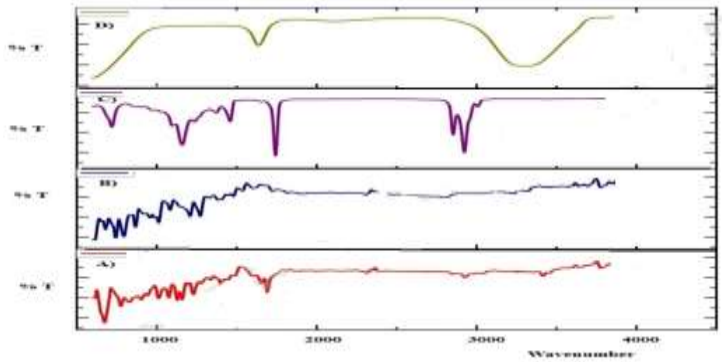


Figure 4: (A) The ATR spectra of RSG showed a significant peak at 3416.83 cm^{-1}, which can be attributed to the moderate intensity resulting from the stretching vibration of the N-H bond in the amine functional group. Additionally, a clear and distinct C=O stretching peak was observed.

(B) For linseed oil, a notable C=O stretching peak was observed, accompanied by a broad, intense absorption peak corresponding to the C-O stretch.
(C) In the case of pyridoxine, a sharp and intense peak associated with the stretching vibrations of hydroxyl groups was detected, along with peaks linked to the stretching modes of C=N and C-N bonds.
(D) The optimal formulation demonstrated a considerably stronger peak intensity and showed a slight deviation compared to the pure RSG compound. This interaction could be due to a reaction between the amine group and a free hydroxyl group present in pyridoxine.

DSC data interpretation
The DSC analysis revealed an endothermic peak at 127.46°C for pure RSG. Additionally, a broader endothermic peak appeared at 216.67°C (Figure 5A). This broadening may be linked to the time-dependent crystallization process, with the observed width possibly arising from molecular reactions occurring at higher temperatures. In Figure 5B, a sharp, well-defined peak at 215.35°C was observed, confirming the purity and presence of pyridoxine. For pure linseed oil, the DSC analysis showed a clear peak at 177.69°C, as seen in Figure 5C. This peak is associated with both the nuclear breakdown and rapid oxidation of the oil at elevated temperatures. The DSC results for the optimal formulation revealed an endothermic peak at 99.62°C (Figure 5D). The inclusion of excipients and oil in this formulation caused a slight decrease in the thermal stability of the drug.

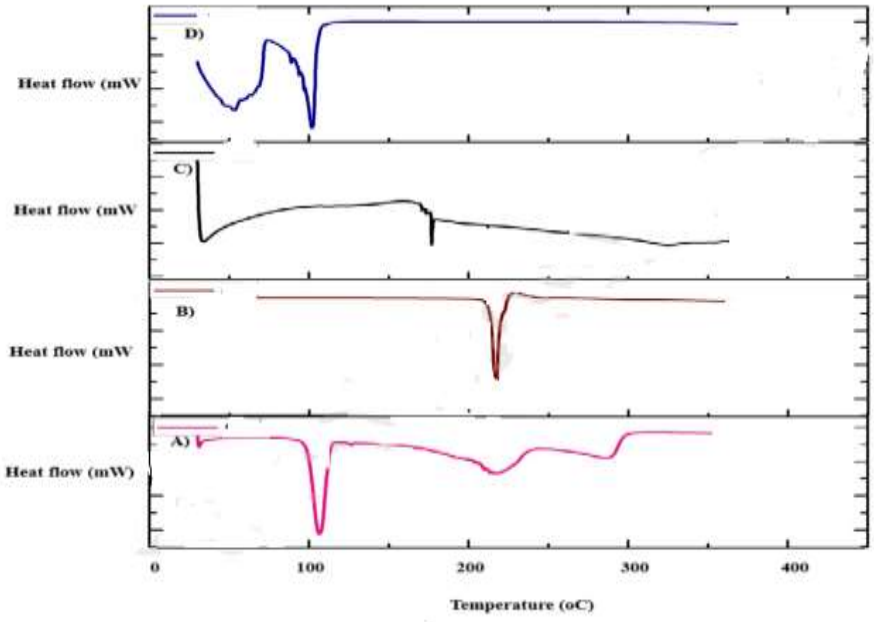


Figure 5: Spectra analysis in DSC thermogram (A) RSG; (B) Pyridoxine; (C) Linseed oil; (D) Optimal formulation

XRD data analysis and interpretation
The main goal of X-ray diffraction (XRD) analysis is to obtain distinct and well-defined peaks from substances with high crystallinity, while minimizing interference from background noise. XRD is used to identify the crystallographic patterns and any changes in a substance after it is combined with different copolymers and excipients. For example, the RSG sample showed prominent peaks at 14.37° and 20.35° (2θ), with intensity levels below 2000. Another peak appeared at 13.56° (2θ). In Figure 6A, additional peaks with intensities below 1100 were identified at 15.80°, 19.38°, 19.84°, 22.63°, and 26.93° (2θ). Pyridoxine demonstrated distinct peaks at 21.18°, 25.41°, and 28.25° (2θ), with intensities under 2400, along with several lower-intensity peaks under 500, as shown in Figure 6B. Pure linseed oil exhibited a broad peak at 22.39° (2θ), with moderate intensity around 1600 (Figure 6C). The broadness of this peak may indicate a lower degree of crystallinity, which could be attributed to the higher concentration of unsaturated α-linolenic acid. The XRD method is frequently used for analyzing crystalline substances. In this study, we also aimed to assess the availability of free RSG in the prepared

formulation. The results presented in Figure 6D show that the optimal formulation displayed two significant peaks at 24.37° and 68.24° (2θ) with reduced intensity. The diffraction pattern showed a decrease in peak intensity, suggesting a decrease in the crystallinity of RSG in the formulation. Furthermore, the broadness of the peak indicated that limited crystallization occurred during the interaction in the solvent solution.

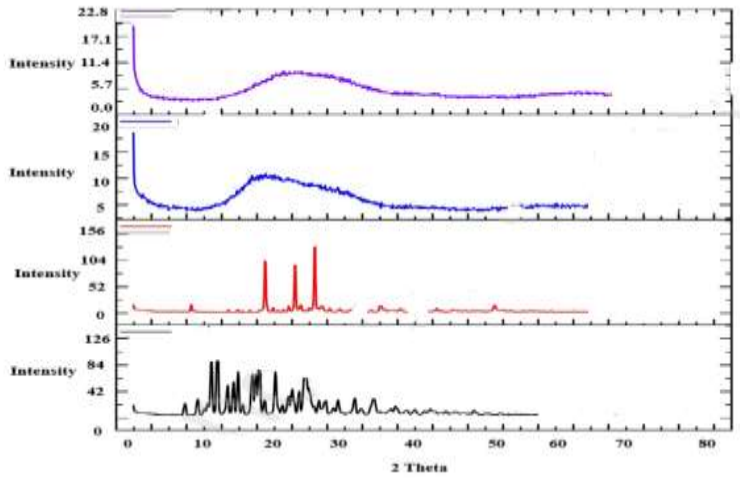


Figure 6: Spectra analysis in XRD diffractogram (A) RSG highlighted two prominent peaks at 15.38 (2Θ degree) and 21.36 (2Θ degree), with an intensity level below 2000; (B) Pyridoxine showed prominent and abrupt peaks at 22.19, 26.42, and 29.26 (2Θ degree) with intensity below 2400; (C) Linseed oil displayed a wider peak at position 23.40 (2Θ degree); (D) Optimal formulation XRD spectrum.

AD treatment progress analysis by MWM model

Before the investigation began, all rat groups, except for Group-I, received intraperitoneal (IP) injections of SP for a duration of 9 days to induce physiological changes resembling those seen in Alzheimer's Disease (AD). Between days 10 and 24, the rats were treated with either "RF6" or "RSG." Following the treatment phase, a 5-day period was designated for the rats to acclimate to their environment. This time allowed the rats to recover from stress and prepared them for the Morris Water Maze (MWM) trial, which lasted for six days.

During the initial phase of the experiment, the rats underwent training to improve their spatial memory. Each rat participated in a swimming task for 60 seconds, with a maximum of five sessions per day. The goal of these sessions was to help the rats learn how to locate a visible platform. The rats were trained in various pool locations during each session. Afterward, the pool was filled with water, and the platform was submerged just 1 cm below the surface. To ensure consistency, the water was made opaque with a non-toxic white paint solution. Over six days, the rats' travel distances to the platform were measured.

Path lengths were recorded with a digital camera and analyzed using one-way ANOVA. The data from the study are presented visually in Figure 7A and 7B. On the first day, the rats in both the RSG and "RF6 nanoemulsion" treatment groups showed similar results. However, as the trial progressed, a reduction in travel distance was observed across all groups. The results indicated that Group-III, which received RSG, showed a significant decrease in travel distance ($p < 0.05$) compared to Group-II. Specifically, Group-II traveled 163 cm to reach the platform, while Group-III, treated with RSG (0.5 mg/kg bodyweight), reduced the distance to 123 cm by the sixth day, indicating progress in recovery from AD.

Rats treated with the optimal formulation, RF6 (RSG in nanoemulsion), showed a further reduction in travel distance. On day 1, Group-IV traveled 218 cm, which significantly decreased to 98 cm by the sixth day. Group-V, treated with a higher dose of RF6 (1.5 mg/kg), demonstrated a similar pattern of reduced travel distance. These results were statistically significant ($p < 0.05$) as determined by ANOVA, as shown in Figure 7C.

The study suggests that RF6 treatments improve cognitive function, particularly learning and memory, in rats with AD. Our findings align with studies by Garcia et al., who demonstrated enhanced blood-brain barrier (BBB) permeability in a model membrane using ferulic acid nanoemulsions, and Klalin et al., who observed increased BBB permeability of lipid nanoemulsions in mice after brain injury. Additionally, our study indicates that the nanoemulsion containing linseed oil was successful in crossing the BBB, leading to improved cognitive performance.

The treatment's progress was notably accelerated, with Group-V's travel distance reducing to 82 cm by the end of the 6-day period. This improvement is likely due to the inclusion of pyridoxine in the formulations.

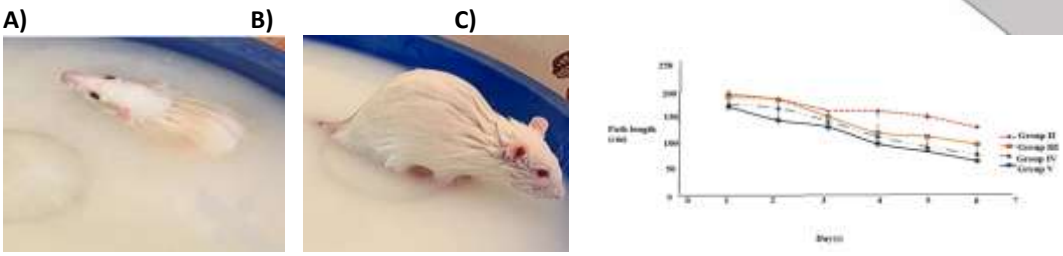


Figure 7: Figure showing: (A) The swimming behavior of treated rats as they navigate towards the platform; (B) A representative image displaying a rat on the platform; (C) The investigation assessed the group behavior of rats, recording the average distance (cm) traveled from each quadrant to the platform, as monitored by a camera. ANOVA analysis revealed statistically significant results.

Conclusion

In this study, nine different formulations were created to develop an effective RSG nanoemulsion for the treatment of Alzheimer's Disease (AD). Pyridoxine was incorporated as a vitamin supplement, while linseed oil served as the oil phase. The resulting nanoemulsion demonstrated prolonged drug release and followed the Higuchi release mechanism. The drug loading (DL) was found to range from 42.99% to 72.36%. The formulation selected for further analysis underwent Scanning Electron Microscopy (SEM), which showed an average globular size of 203.4 nm. The chosen formulation, labeled "RF6," exhibited a high stability, with a zeta potential of -16.7 mV. X-ray diffraction analysis indicated a decrease in peak intensity, suggesting a reduction in the crystallinity of RSG. Fourier Transform Infrared (FTIR) spectroscopy revealed a distinct peak at 3304.05 cm-1, corresponding to the stretching of the N-H bond in the amine group of RSG. This peak was more intense and slightly shifted compared to the pure RSG molecule, indicating a potential interaction between the amine group and a free hydroxyl group present in pyridoxine.

The results of the study showed that increasing the concentration of linseed oil led to higher encapsulation efficiency (EE). On the other hand, higher pyridoxine concentrations and its water solubility in the "RF5" formulation resulted in a lower EE. The Morris Water Maze (MWM) model demonstrated an improvement in AD symptoms for subjects receiving "RF6" treatment. Statistically significant results ($p<0.05$ by ANOVA) were observed for Group-III therapy with RSG, as compared to Group-II. Additionally, treatments in Group-IV and Group-V showed a reduction in the distance traveled (98 cm and 82 cm, respectively, on the 6th day) before reaching the hidden platform. These findings suggest that the combination of RSG, pyridoxine, and linseed oil not only facilitates crossing the blood-brain barrier but also reduces trip distance in just six days. Further studies on toxicity using appropriate animal models can be explored.

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