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RESEARCH ARTICLE

Radiolabeling, Characterization, and *In Vitro* Cell Culture Studies of Donepezil-Loaded Niosome Formulations For Brain Imaging

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SUMMARY

Dementia has posed a significant public health challenge for aging populations in developed countries for many years. Alzheimer's Disease (AD) is the most prevalent cause of dementia, marked by cognitive and behavioral irregularities resulting from cholinergic dysfunction, a progressive and irreversible neurodegenerative disorder. Restoring cholinergic neurotransmission can alleviate impaired cognitive and behavioral symptoms in AD patients. Donepezil (DNP) is an acetylcholinesterase inhibitor utilized to treat mild to moderate AD symptoms. Niosomes, non-ionic surfactant vesicles, present various benefits as drug delivery systems, including stability, non-immunogenicity, permeation potential, and controlled release ability. In this research, we prepared, characterized, radiolabeled with 99mTc, and evaluated DNP-loaded niosome formulations as radiopharmaceuticals for brain imaging agents. The findings suggest that this innovative radiopharmaceutical ([99mTc]Tc-formulation) has the potential to serve as a promising alternative imaging agent for neurological disorders.

Key Words: *Donepezil, Tc-99m, niosome, cytotoxicity, radiopharmaceutical*

Beyin Görüntüleme İçin Radyolojik İşaretlenmiş Donepezil Yüklü Niozom Formülasyonlarının Karakterizasyonu ve In Vitro Hücre Kültürü Çalışmaları

ÖZ

Demans, uzun yıllardan beri gelişmiş ülkelerdeki yaşlanan nüfus için önemli bir halk sağlığı sorunu teşkil etmektedir. Alzheimer Hastalığı (AH), ilerleyici ve geri dönüşü olmayan bir nörodejeneratif hastalık olan kolinerjik fonksiyon bozukluğundan kaynaklanan bilişsel ve davranışsal düzensizliklerle karakterize edilen demansın en yaygın nedenidir. Kolinerjik nörotransmisyonun yeniden sağlanması, AD hastalarında bozulmuş bilişsel ve davranışsal semptomları hafifletebilir. Donepezil (DNP), hafif ila orta dereceli Alzheimer semptomlarını tedavi etmek için kullanılan bir asetilkolinesteraz inhibitörüdür. Niozomlar, stabilite, non-immunojenite, permeasyon potansiyeli ve kontrollü salım yeteneği gibi bir dizi avantaj sunan non-iyonik yüzey aktif madde vezikülleridir ve ilaç taşıma sistemleri olarak kullanılabilirler. Bu araştırmada, DNP yüklü niozom formülasyonları hazırlandı ve karakterize edildi. Daha sonra 99mTc ile radyoişaretlendi ve beyin görüntüleme ajanları olarak kullanılmak üzere değerlendirildi. Elde dilen bulgular sonucunda, bu yenilikçi radyofarmasötiğin ([99mTc]Tc-formülasyonun) nörolojik bozukluklar için umut vadeden alternatif bir görüntüleme ajanı olarak hizmet etme potansiyeline sahip olduğunu görüldü.

Anahtar Kelimeler: *Donepezil, Tc-99m, niozom, sitotoksisite, radyofarmasötik*

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INTRODUCTION

Alzheimer's disease (AD) is the leading cause of dementia, accounting for 70-80% of diagnosed cases of neurodegenerative brain disease (Pratik et al., 2021). It has emerged as a growing global health concern, quickly becoming one of the most expensive, lethal, and burdening diseases of this century (Van der Flier et al., 2023). The number of people worldwide living with dementia is predicted to reach 74.7 million in 2030 and 131.5 million in 2050 (Prince, 2015). The pathology of AD is characterized by the accumulation of misfolded proteins, inflammatory changes, and oxidative stresses, resulting in the loss of synaptic contacts and neuronal cell death (Ahmed et al., 2016). AD causes memory loss and deterioration of other cognitive abilities that interfere with daily life (Breijyeh & Karaman, 2020). The early signs of the disease include a decline in non-memory aspects of cognition, such as finding the right words, forgetting recent events, trouble understanding visual images and spatial relationships, and impaired reasoning or judgment. As the disease progresses, symptoms such as increased confusion, behavior changes, impaired mobility, hallucinations, and seizures become more severe. Although the disease's progression may differ for each individual, most people live between four and eight years after diagnosis (Lane et al., 2017; Rasmussen & Langerman, 2019). While there is no definitive cure for Alzheimer's disease, certain medications can temporarily slow the worsening of dementia symptoms. The United States Food and Drug Administration (FDA) has approved two types of drugs to treat the symptoms of Alzheimer's disease: Acetylcholinesterase (AChE) inhibitors [i.e., Donepezil (DNP), Rivastigmine, Galantamine] and N-methyl-D-aspartate (NMDA) antagonists (i.e., Memantine). DNP is a new class of AChE inhibitor having an N-benzyl piperidine and an indanone moiety that shows longer and more selective action for the treatment of mild to moderate AD (Sugimoto, 2001; Nayak et al., 2020). It is effective against the three major domains of AD symptoms: functional ability, behavior, and cognition, and can be administered orally or transdermally (Knowles, 2006). Early and accurate diagnosis of AD is fundamental for subsequent patient management. Early diagnosis enables patients to seek early intervention with symptomatic treatment, lifestyle changes to maintain quality of life, and risk-reduction strategies that can clinically reduce cognitive, functional, and behavioral decline (Porsteinsson et al., 2021). AD is diagnosed with physical and neurological exams, blood tests, mental status and neuropsychological testing, and brain imaging. Researchers are working on new brain imaging techniques to diagnose better and track the progress of AD. Brain imaging is a complex field that faces several challenges. With the increasing number of available imaging modalities, specialized agents are becoming more prevalent. These agents are designed to address the limitations of current imaging techniques and provide more accurate and detailed information about the brain. As a result, efforts towards developing and utilizing such agents are growing rapidly. Despite these advancements, there are still significant challenges to be overcome in daily clinical practice (Ali et al., 2016; Subramaniam, 2017). Brain SPECT agents are a type of diagnostic imaging agent that are used to examine the brain for various diseases. These agents include ^{99m}TcO4-, [^{99m}Tc] DTPA, ^{201}TI, and [^{67}Ga]citrate. They are not taken up by normal brain cells but can penetrate tumor cells due to the altered blood-brain barrier. This makes them useful for identifying brain diseases. On the other hand, SPECT perfusion agents such as [^{123}I] IMP, [^{99m}Tc]HMPAO, and [^{99m}Tc]ECD diffuse into the normal brain. These agents are fat-soluble and are effective in detecting various cerebrovascular diseases, including stroke, Parkinson's disease, Huntington's disease, epilepsy, dementia, and psychiatric disorders. In summary, brain SPECT agents and SPECT perfusion agents are both important tools for diagnosing brain diseases. Brain SPECT agents are useful for detecting diseases in the altered blood-brain barrier, while SPECT perfusion agents effectively detect cerebrovascular diseases in the normal brain (Ono et al., 2014; Yomo & Oguchi, 2017). However, newer

radiopharmaceuticals are needed. Niosomes are fascinating colloidal systems comprising synthetic surfactants that are non-ionic. These surfactants possess the unique ability to self-assemble and form bilayer structures that enclose one or more aqueous compartments within. This remarkable feature makes them ideal for targeted drug delivery applications, where precise and controlled release of therapeutic agents is required (Bragagni et al., 2014). They exhibit a wide range of desirable properties, including tunable vesicle size, high loading capacity, sustained drug release, targeted drug delivery, osmotic stability, ease of fusogenicity, and intracellular drug release (Keerthana et al., 2022). Niosomal formulations have prolonged circulation in the bloodstream, allowing for enhanced targeted action due to their non-ionic surfactants. They enable the targeted delivery of various types of drugs, thanks to their versatile structure (Umbarkar, 2021). Niosomes have also been suggested as a tool to deliver drugs to the brain due to the high level of cerebral glucose uptake (Bragagni et al., 2012). Niosomal formulations are among the most suitable systems for transporting the drug to the desired area. Research on radiolabeling studies with niosomal formulations for use in the diagnosis of diseases is becoming widespread. Developing an effective and feasible method for radiolabeling niosomal formulations will allow these drug delivery systems to be used with nuclear medicine imaging methods. In this study, we prepared and characterized DNP-loaded niosome formulations. These formulations were radiolabeled with ^{99m}Tc and evaluated as radiopharmaceuticals for brain imaging agents.

MATERIAL AND METHODS

The laboratory procured Cholesterol (Chol), Span 85, and Chloroform from Sigma-Aldrich in the United States, while the Nuclear Medicine Department of Ege University provided the Molybdenum-99 (^{99}Mo)/^{99m}Tc generator used to elute [^{99m}Tc] TcO_4^-. For cell culture experiments, Gibco Invitrogen in Grand Island, NY, supplied the necessary reagents and supplies. HT-22 was chosen and obtained from the American Type Culture Collection (ATCC) for cell culture studies. All other reagents were of analytical grade unless otherwise stated and obtained from commercial sources.

Preparation of niosome formulations

The process of creating niosome formulations involved utilizing the thin-film hydration technique (TFH), (Arunothayanun et al., 2000; Nayak et al., 2020). The first step was dissolving Span 85 and cholesterol in 10 mL of chloroform within a round-bottom flask. The next step involved evaporating the chloroform under vacuum at 60°C using a rotary evaporator, which resulted in a thin film (Buchi Rotavapor®, B-100). Afterward, the thin film was hydrated for 45 minutes with PBS (pH 7.4) under rotation at 65°C (Akbarzadeh et al., 2021). Once hydrated, the formulation was sonicated using Bandelin Sonopuls® HD 2070. The formulations were then stored in the refrigerator at 4°C until use. For DNP-loaded formulations, 15 mg of the active agent was dissolved in chloroform along with Span 85 and cholesterol. More information about the composition and structure of the niosome formulations was shown in (Table 1).

Table 1. The composition of niosome formulations

Formulation Code	Span 85: Cholesterol Ratio	Cholesterol (mg/mL)	DNP (mg/mL)
F1	1	15	-
F2	1	30	-
F3	1	45	-
F4	0.5	15	-
F5	0.5	30	-
F6	0.5	45	-
F3+DNP	1	45	1.5

Characterization of niosome formulations

Particle size, polydispersity index, and zeta potential

At room temperature, the Malvern Zetasizer (Malvern Nano ZS, Malvern, UK) was used to determine the mean diameters, polydispersity index values, and Zeta potential of niosomes. The measurements were performed in triplicates, and the results were presented as the mean value ± SD.

Viscosity studies

The viscosity of formulations was measured using a sine-wave viscometer (Vibro SV-10) at a measurement range of 10–10.000 mPa·s. The viscosity was measured by placing the sample fluid between the sensor plates of the viscometer and measuring it at 25°C.

Injectability studies

The injectability of the niosome formulation was measured using the TA-XT Plus in compression mode and an injectability probe (Universal Syringe Rig) equipped with a 30 kg load cell. To conduct the test, a 2 mL syringe filled with the formulation was placed in the metallic support of the probe. A constant force of 0.5 N was applied to push the plunger. The force-time curves were used to calculate the work and force required to expel the formulation from the syringe. The area under the resulting curve was used to determine the work of expulsion (Rungseevijitprapa & Bodmeier, 2009). The study was performed at room temperature (25°C), and measurements were taken in triplicates. The results were presented as the mean value ± standard deviation.

Encapsulation efficiency (EE)

The encapsulation efficiency of the formulation was determined by using a dialysis bag (Spectrum Ltd., Shanghai, China) with 8000–12,000 Da of molecular weight. For that, 1 mL of formulation was put into dialysis bags. The samples were ultra-centrifuged at 5000 rpm at room temperature and filtered using a cellulose nitrate membrane. The concentration of donepezil was analyzed by spectrophotometry.

Cell culture studies

For cytotoxicity studies, the HT-22 cells were utilized. The cells were grown in a cell medium of DMEM, which was supplemented with 10% fetal bovine serum. To prepare the cell monolayers, 1 × 105 cells were seeded onto 96-well plates. The cell culture was maintained at 37°C, 90% humidity, and 5% CO_2.

***In vitro* cytotoxicity studies**

The purpose of the study was to determine the effect of different concentrations of DNP solutions on the cytotoxicity of HT-22 cells, which are similar to hippocampal neuronal cells. The HT-22 cells were cultured in DMEM, which was supplemented with 10% fetal bovine serum, 0.5 mg/mL glutamine/penicillin-streptomycin, and then incubated at 37°C with 5% CO_2. The experiment involved using the 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay to analyze the cytotoxicity of DNP solutions with varying concentrations (0.05 mg/mL, 0.1 mg/mL, 0.5 mg/mL, 1.5 mg/mL, 2 mg/mL, 5 mg/mL) on HT-22 cells. To seed the cells, 1 × 10^6 cells/well in 1 mL DMEM were added to 96-well flat-bottom plates. DNP was solubilized in DMEM (1 mg/mL) and then incubated at different concentrations (0.05 mg/mL, 0.1 mg/mL, 0.5 mg/mL, 1.5 mg/mL, 2 mg/mL, 5 mg/mL). The DNP solutions were added to the well plates and incubated for 24, 48, and 72 hours. After the incubation period, 100 µL of MTT solution (5 mg/mL) was added to each well and incubated at 37°C. Following that, the culture medium was removed, and 200 µL of dimethyl sulfoxide was added to dissolve the MTT formazan crystals. The absorbance was read at 570 nm using a microplate reader. Finally, the IC50 (the concentration that inhibits 50% of cell viability) was calculated using the "GraphPad Prism" and the "ne-site total binding" algorithm programs (Gundogdu et al., 2012).

Radiolabeling studies

To determine the best radiolabeling conditions, we conducted experiments with varying amounts of stannous chloride as the reducing agent. The formula-

tions were radiolabeled by reacting with [^{99m}Tc]TcO_4^- at room temperature (25°C) and pH 7.0. We added different amounts of the reducing agent solution (50, 250, and 1000 μg mL^{-1} stannous chloride in distilled water) to the formulations dispersed in 0.5 mL of 0.9% sodium chloride solution (SF). We then carried out the radiolabeling process using freshly eluted 37 MBq/0.1 mL [^{99m}Tc]TcO_4^- and shook the resulting radiolabeled formulations for 60 seconds before incubating them for 15 minutes. To analyze the radiolabeling efficiency of the formulations, we used Radio Thin Layer Chromatography (RTLC) (Gundogdu et al., 2015; Karpuz et al., 2021). Additionally, we radiolabeled the blank formulation using the same procedure and used it as a control.

Quality Control by Radio Thin Layer Chromatography (RTLC)

We conducted RTLC to verify the effectiveness of the labeling process of the formulations. The experiment involved using 2 μL of the labeled formulations and acetone (Sigma-Aldrich) as the mobile phase on Whatman paper no. 1. The RTLC was performed for 15 minutes. Afterward, we analyzed the radioactivity of the strips using a TLC scanner (Bioscan AR 2000).

***In vitro* stability studies**

An evaluation was conducted to determine the stability of [^{99m}Tc]Tc-formulations in cell medium (McCoy's 5A supplemented with 10% fetal bovine serum). To conduct the experiment, 3.7 MBq of [^{99m}Tc]Tc-formulations were mixed with 0.4mL SF and incubated at 37°C. Radiolabeling efficiency studies were conducted during the experiment to determine the stability of the mixture.

RESULTS AND DISCUSSION

The results of this study highlight the potential of niosomes as a valuable tool for treating and imaging brain diseases, including AD. The ability of niosomes to cross the blood-brain barrier and deliver drugs to the central nervous system is significant in improving therapeutic outcomes. Niosomes can enhance the solubility and stability of pharmaceutical molecules, allowing for better drug delivery and controlled release. This is particularly important in the case of brain diseases where the blood-brain barrier is a significant obstacle to drug delivery. DNP-loaded niosome formulations have been developed as potential nanocarriers for nuclear imaging techniques. The study confirms that the radiolabeled formulations have favorable radiolabeling efficiency and stability, showing promise as radiolabeled nanocarriers for use in nuclear imaging and further studies.

Preparation and characterization of niosome formulations

Niosomes are a type of vesicular drug delivery system mainly composed of non-ionic alkyl or polyglycerol etheric surfactant and cholesterol in an aqueous medium. These drug delivery systems are formulated with non-ionic amphiphiles in specific aqueous solutions using self-assembly technology to create the vesicles.

Niosomes have emerged as a safe and effective option for drug delivery, primarily due to their composition of biocompatible, biodegradable, non-toxic, and non-immunogenic materials. This makes them ideal for use in numerous clinical applications. Niosomes offer several advantages, such as stability, low cost, ease of formulation, and scale-up potential. They can effectively encapsulate hydrophilic, lipophilic, and amphiphilic drugs, allowing for controlled and sustained release of drugs. Furthermore, niosomes have a higher bioavailability than conventional dosage forms and can protect drugs from biological enzymes and acids (Verma et al., 2023). They also show great promise in targeting drugs to the central nervous system and overcoming the blood-brain barrier (Gharbavi et al., 2018). Overall, niosomes represent an excellent option for drug delivery in the clinical setting, with the potential for significant therapeutic benefits. Niosomes have emerged as a promising drug delivery system for treating neurological disorders, such as epilepsy, seizures, trauma, Parkinson's disease, multiple sclerosis, dementia, and AD. They can potentially

reduce AD pathogenesis and offer a more stable and beneficial drug delivery system for the brain.

Various methods have been used to produce niosomes, such as sonication, ether injection, microfluidization, TFH, and bubble technique. The TFH technique is a straightforward and practical approach for niosome preparation. In this method, surfactants and cholesterol are dissolved in an organic solvent within a round-bottom flask. Subsequently, the organic solvent is removed through rotary evaporation and vacuum to form a thin layer on the inner surface of the flask. Then, a solution, such as water or PBS, is introduced, and the dried layer is rehydrated above the surfactant's transition temperature to form niosomes (Zinatloo-Ajabshir & Zinatloo-Ajabshir, 2019). Niosomes can be either uni-lamellar or multi-lamellar vesicles composed of non-ionic surfactants, cholesterol, and ionic surfactants used to prevent formulation aggregation. We prepared niosome vesicles with non-ionic surfactant and cholesterol. Surfactant (Span 85) and cholesterol ratios were selected as 0.5:1 and 1:1, and the lipid-to-DNP ratio was 10-30.

Cholesterol is a vital component in maintaining the stability and rigidity of niosomes. However, an excessive amount of cholesterol can lead to reduced fluidity in vesicles, impeding the penetration and permeability of drugs (Umbarkar, 2021). By adjusting the ratio of cholesterol to surfactant, one can effectively study the influence of cholesterol on factors such as nanoparticle size, zeta potential, size distribution, and entrapment efficiency (Agarwal et al., 2004). Within (Table 2), the effects of varying ratios of cholesterol to surfactant on the size and zeta potential values of niosomes are displayed. By adjusting this ratio, niosomes with a range of particle sizes can be generated. Notably, the F3 formulation exhibited the lowest particle size and polydispersity index value compared to the other niosomes. This finding suggests that F3 is the ideal option for additional investigation.

The PDI measures the degree of uniformity in particle size. It is calculated as the ratio of the standard deviation to the mean particle size. A PDI value of 0.3 or less indicates a high degree of uniformity in particle size. This metric is an important consideration in various fields, such as pharmaceuticals, material sciences, and biotechnology, where particle size uniformity is crucial to product performance and efficacy. The PDI value of niosome formulations was measured as 0.304 ± 0.040, indicating a uniform particle size distribution (Shadab et al., 2014; Asmari et al., 2016; Yasir et al., 2017). The characterization of the DNP-loaded niosome formulation demonstrated a uniform particle size of 199.30±4.86 nm, which is considered ideal for niosome formulations. It was observed that the incorporation of the active ingredient (DNP) into the niosome nucleus did not significantly change the particle size but led to an increase in particle distribution relative to blank niosome particles. The increase in PDI value was significant ($p < 0.05$) compared to blank niosomes, however, the PDI value is still around 0.3 which shows a high degree of uniformity.

The zeta potential magnitude indicates the colloidal system's potential stability. When all suspended particles have a significantly positive or negative zeta potential, they repel each other, preventing particle aggregation. Conversely, if the particles have low zeta potential values, there is no force to impede their tendency to aggregate. The importance of the zeta potential is that its values can be correlated with the electrophoretic movement of the particles and the stability of the formulation. Typically, particles with zeta potentials above +30 mV or below -30 mV are generally considered stable. The zeta potential value provides information about the charge on the niosomes. A sufficiently high zeta potential value within the range of +30 mV to -30 mV contributes to stability by preventing particle aggregation (Shadab et al., 2014; Asmari et al., 2016; Yasir et al., 2017). The zeta potential value of F1-F6 are presented in Table 2 and formulations F1-F6 exhibited a negative charge ranging from (-31.8) to (-33.8). The zeta potential values of F3 and F3+DNP were found to be -32.6 ±

0.862 mV and -15.4 ± 0.656 mV, respectively. It can be seen that DNP incorporation into niosomes caused a significant increase in zeta potential that can be attributed to the preferential adsorption of counter ions or hydrogen ions on the niosome shell with the addition of an active substance. (Behbahani et al., 2017). Also, results showed that Zeta potential values didn't change significantly by changing cholesterol to surfactant ratios. The optimized niosome formulation demonstrated a uniform particle size of 199.30±4.86 nm, a PDI value of 0.304 ± 0.040, and a zeta potential value of -15.4 ± 0.656 mV, indicating good stability.

Table 2. Particle size, polydispersity index, and zeta potential values of formulations

Formulation Code	Particle size (d.nm) ± SD	Polydispersity Index ± SD	Zeta Potential (mV) ± SD
F1	318.10 ± 3.960	0.210 ± 0.008	-33.6 ± 2.166
F2	259.10 ± 3.470	0.202 ± 0.041	-33.8 ± 2.307
F3	198.70 ± 9.407	0.148 ± 0.016	-32.6 ± 0.862
F4	216.90 ± 9.756	0.183 ± 0.096	-31.8 ± 1.686
F5	227.30 ± 7.778	0.159 ± 0.060	-32.6± 1.069
F6	235.80 ±5.798	0.149 ± 0.044	-34.2 ± 2.574
F3+DNP	199.30± 4.860	0.304 ± 0.040	-15.4 ± 0.656

pH Measurements

It is crucial to maintain the appropriate pH value in parenteral formulation to ensure its safety and efficacy. For intravenous (i.v.) and intramuscular injections, the target pH should be as close as possible to the physiological pH, within a range of pH 2-11. For subcutaneous injection, the acceptable range is pH 4-9 due to potential irritation issues. The pH value of the F3 niosome formulation has been measured as 6.94 ± 0.07, which falls within the safe range for i.v. administration. Therefore, it can be concluded that the F3 niosome formulation is safe to use for i.v. administration.

Viscosity studies

Viscosity plays a crucial role in ensuring the smooth flow and distribution of drugs during the injection process. Therefore, appropriate viscosity is essential for parenteral formulations. The viscosity of the DNP-loaded niosome formulation and serum physiological (SP) were determined to be 0.820 ± 0.226 cP and 0.850 ± 0.212 cP, respectively. The results showed that there was no statistically significant difference between the viscosity of the niosome formulation and SP. This indicates that the niosome formulation exhibits similar viscosity characteristics to SP, which is an important aspect to ensure the smooth flow and distribution of the drug during the injection process.

Injectability study

Injectability is an important aspect to consider when optimizing the usability and patient comfort of parenteral formulations. It refers to the ability of a formulation to be successfully administered by a syringe. To test the injectability of the niosome formulation, it was compared with serum physiological (SP) at 25°C. The work required to expel niosome formulation from the syringe was determined to be 7.772 ± 1.167 N.sec, and it was found to be statistically similar to the injectability of SP, as seen in (Table 3) and (Figure 1). The results of the injectability studies were found to be in accordance with the results of the viscosity studies. This indicates that the injectability values of the niosome formulation are suitable for parenteral administration, ensuring successful and comfortable receipt of the drug.

Table 3. The injectability value of formulations

Formulation	Stiction (N)	Plateau Force (N)	Work (N.sec)
F3 + DNP	3.84 ±0.11	2.58±0.40	7.772±1.167
Serum Physiological	4.22±1.09	2.06±0.56	7.145±0.986

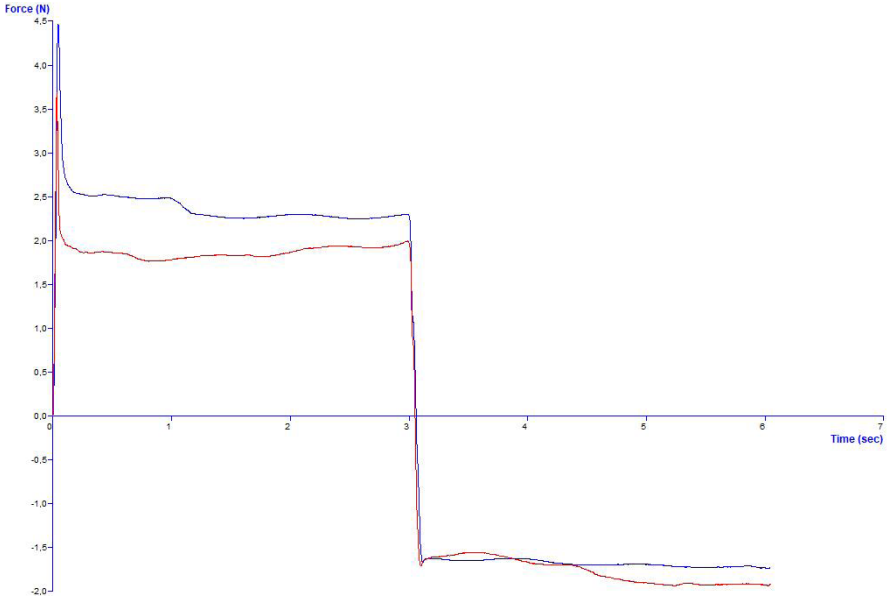

Figure 1. Force time profiles from injectability testing using TPA (Blue line: F3+DNP, Red line: SP)

Encapsulation efficiency (EE)

The calibration curve of the active ingredient DNP is shown in Figure 2.

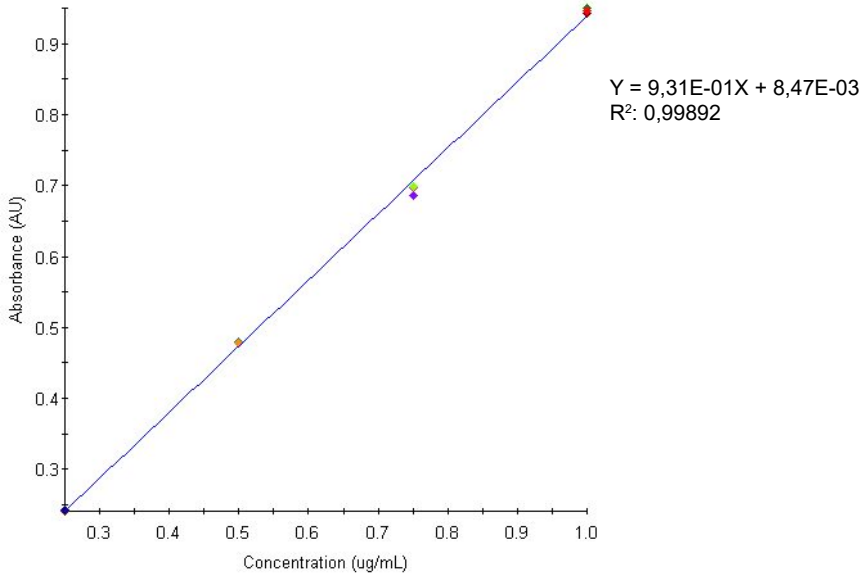

Figure 2. Calibration curve of donepezil

The encapsulation efficiency of donepezil in the optimized formulation (F3) was found to be 80%. The precise management of a drug delivery system hinges upon the EE of the formulation. The desired EE values are crucial in avoiding dose-dependent side effects, which ultimately enhance patient adherence to treatment (Amasya et al., 2019). According to this study, the developed formulation successfully attained the intended EE value.

Radiolabeling studies

[^{99m}Tc]TcO_4^- is a highly useful radioactive tracer that is generated by the 99Mo/99mTc generator. However, in its stable form, it exists as a pertechnetate in the +7 oxidation state, which makes it unable to bind to any compound directly. To facilitate its binding to a pharmaceutical compound, reducing agents are employed to convert it to a more reactive +4/+5 oxidation state (Elitez et al., 2018; Wu et al., 2020). Among the commonly used reducing agents for [^{99m}Tc]TcO_4^- radiopharmaceuticals are stannous salts. For this study, stannous chloride was utilized as a reducing agent to label the niosome formulation with [^{99m}Tc]TcO_4^- directly. The study examined the impact of different amounts of stannous chloride (50, 250, and 1000 µg mL^{-1}) on the radiolabeling process. The results, which demonstrate the radiolabeling efficiency of the niosome formulation, are presented in (Figure 3).

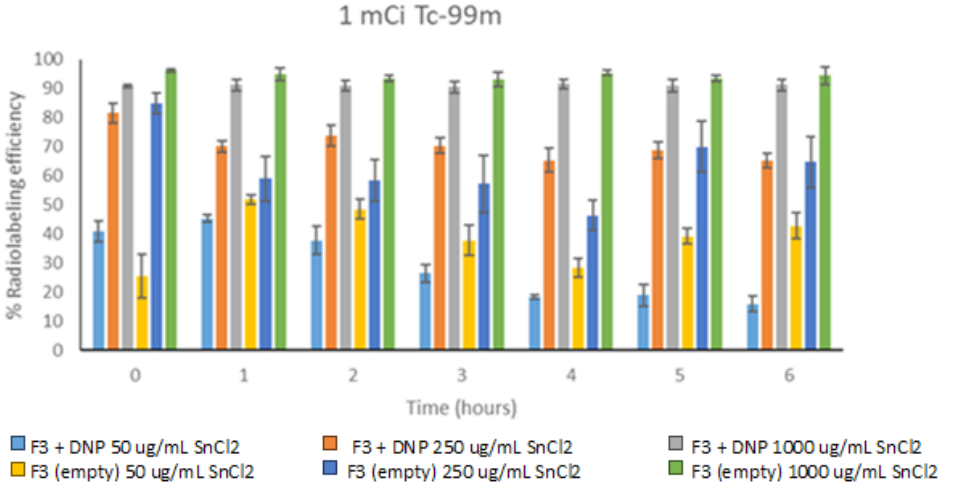

Figure 3. Radiolabeling efficiency % of formulations in different stannous chloride concentrations

In vitro stability studies

Cold kits are dissolved in SF and labeled with [^{99m}Tc]TcO_4^- in nuclear medicine before being administered to patients. Likewise, in cell culture studies, radiolabeled formulations are incubated with a cell medium for a designated time. Hence, it's crucial to examine the stability of radiolabeled formulations in the cell medium. (Figure 4) illustrates the outcomes of this examination.

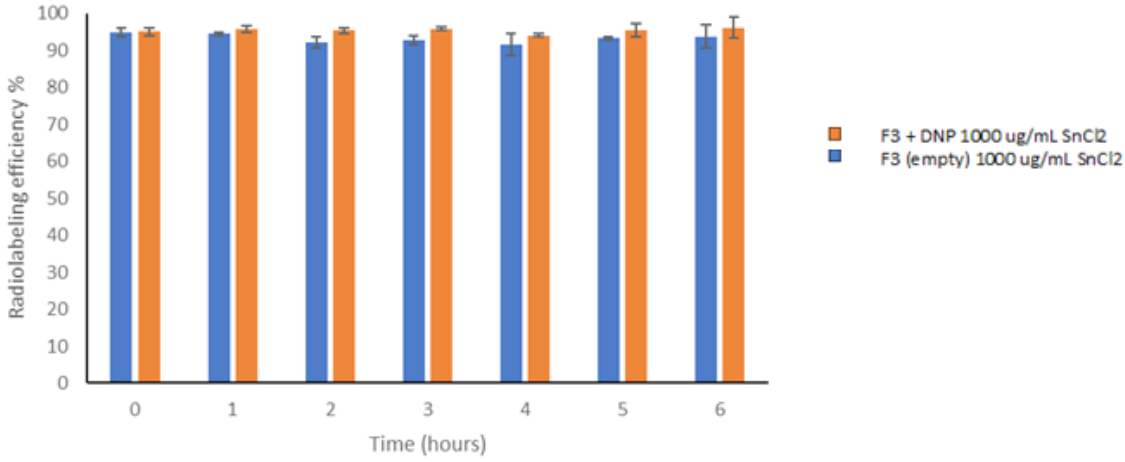

Figure 4. *In vitro* stability of radiolabeled formulations

Based on the findings displayed in Figure 3, the radiolabeling efficiency of the formulations remained consistent for up to 6 hours after the radiolabeling process. These results strongly suggest that the in vitro stability and labeling efficiency are well above 90% while in a cell medium. With this level of stability, the [^{99m}Tc]Tc-formulations can serve as an imaging agent for a minimum of 6 hours after preparation.

***In vitro* cytotoxicity studies**

The objective of this study was to examine the cytotoxicity of DNP solutions with varying concentrations (0.05 mg/mL, 0.1 mg/mL, 0.5 mg/mL, 1.5 mg/mL, 2 mg/mL, and 5 mg/mL) on HT-22 cells. As shown in (Figure 5), the DNP solution with a concentration of 1.5 mg/mL displayed cell viability of over 95%, while the other concentrations indicated over 85% cell viability after 72 hours of treatment. This suggests that the DNP solution with a concentration of 1.5 mg/mL is less toxic than the other concentrations. Using GraphPad Prism, the IC50 value of DNP for HT-22 cells was calculated and determined to be 24.54 ± 1.44 µM (Figure 6). These results suggest that DNP concentrations equal to or less than 1.5 mg/mL and below the IC50 value applied to HT-22 cells are unlikely to cause toxicity. Therefore, it can be considered safe and effective as a drug delivery system, making it a suitable option for administration.

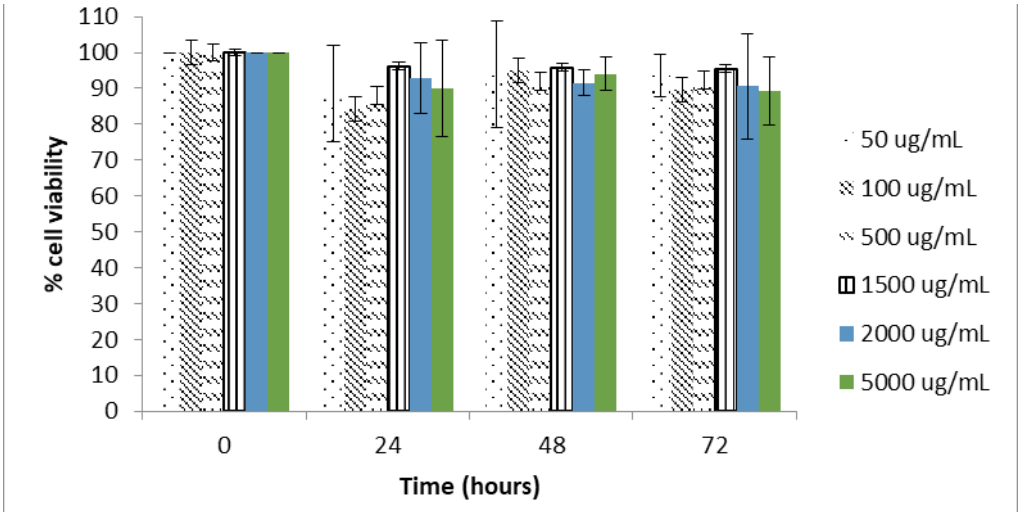

Figure 5. Cell viability % of DNP solution at different concentrations

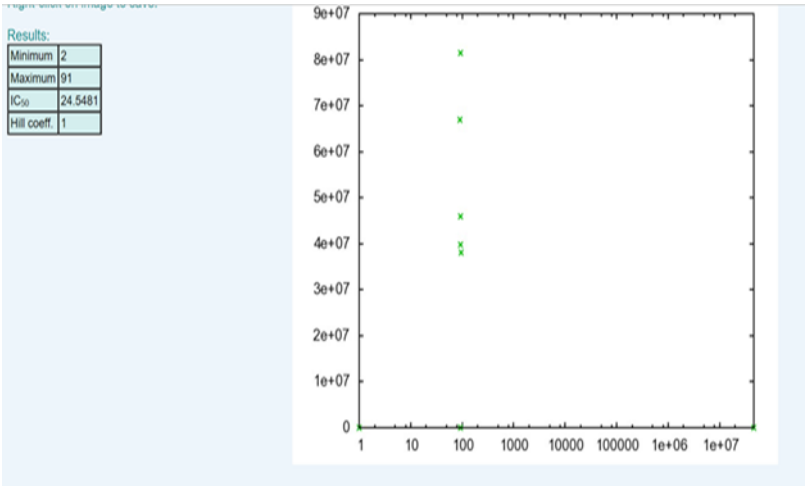

Figure 6. IC50 value of DNP in DMEM (IC50 (24,54 μM)

CONCLUSION

The purpose of this investigation was to create a novel radiopharmaceutical capable of imaging various regions of the brain. To accomplish this, DNP HCl niosomes were fabricated using the TFH technique, and different surfactant/cholesterol ratios (0.5, 1) were evaluated. The cholesterol-to-surfactant ratio was identified as a crucial factor in determining the size, zeta potential, and size distribution of the niosomes. Following multiple trials, the F3 niosome was selected as the optimal formulation and radiolabeled with ^{99m}Tc. The labeled compounds remained stable for up to 6 hours at room temperature and during experimentation in cell media. The highest radiolabeling efficiency was achieved when the formulations included 1000 μg of stannous chloride and 37 MBq [^{99m}Tc] $NaTcO_4^-$ at pH 7.0 with a 15-minute incubation time.

In summary, the newly developed radiopharmaceutical, [^{99m}Tc]Tc-formulation, displays potential as an alternative imaging agent. Additionally, the findings propose that niosomes could be a promising factor for the treatment and imaging of brain diseases, particularly Alzheimer's disease. Further research and development in this field are necessary to examine the full potential of niosomes and the utilization of [^{99m}Tc] Tc-formulation as an effective and targeted delivery system and imaging agent for neurological disorders.

AUTHOR CONTRIBUTION STATEMENT

Conception (EO, HA, ZS, EAG), Design (EO, HA, ZS, EAG), Data collection (EO, HA, ZS, EAG), writing manuscripts (EO, HA, ZS, EAG), supervision (EO, HA, ZS, EAG)

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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