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Article

Investigation on formulation parameters of donepezil HCl loaded solid lipid nanoparticles

Gizem Rüya Topal[1,2], Berrin Küçüktürkmen[2], Umut Can Öz[2], Erva Özkan[3], Filiz Bakar-Ates[3], Asuman Bozkır[2*]

[1]Department of Pharmaceutical Biotechnology, Gulhane Faculty of Pharmacy, University of Health Sciences, Ankara, Turkey, [2]Department of Pharmaceutical Technology, Faculty of Pharmacy, Ankara University, Ankara, Turkey, [3]Department of Biochemistry, Faculty of Pharmacy, Ankara University, Ankara, Turkey

Donepezil-HCl is a member of the acetylcholinesterase inhibitors that is indicated for the symptomatic treatment of Alzheimer's disease (AD) and has many side effects. In this study, to reduce the side effects of Donepezil-HCl and increase the penetration of the drug through the blood-brain barrier, we aimed to design a solid lipid nanoparticle (SLN) formulation. The effects of the different formulation parameters, such as homogenization speed, sonication time, lipid and drug concentration, surfactant type and concentration, and volume of the aqueous phase, were assessed for optimization. The particle size and PDI increased with increasing lipid concentration but decreased with increasing amounts of surfactant (Tween 80) and co-surfactant (lecithin). When the homogenization rate and sonication time increased, the particle size decreased and the encapsulation efficiency increased. The optimized formulation exhibited particle size, PDI, encapsulation efficiency, and zeta potential of 87.2±0.11 nm; 0.22±0.02; 93.84±0.01 %; -17.0±0.12 mV respectively. The in vitro release investigation revealed that approximately 70% of Donepezil-HCl was cumulatively released after 24 hours. TEM analysis proved that spherical and smooth particles were obtained and formulations had no toxic effect on cells. The final optimized formulation could be a candidate for Donepezil-HCl application in Alzheimer's treatment with reduced side effects and doses for patients.

Keywords: Donepezil HCl. Solid lipid nanoparticles. Alzheimer's Disease. Homogenization-sonication technique. Tripalmitin.

INTRODUCTION

Alzheimer's disease (AD) is the most common type of dementia. Consequently, it causes memory deformation, cognitive decline, and behavioral dysfunction. Today, nearly 50 million people live with this disease, and it is expected that the number of patients will triple by 2050 all around the world (Arvanitakis, Shah, Bennett, 2019). Although AD has been known for many years, only four acetylcholinesterase (AChE) inhibitors and Memantine are being used as approved drugs for treatment. These drugs provide symptomatic treatment but do not change the course of the disease. Depletion of acetylcholine is a very common effect in AD patients. Considering the cholinergic hypothesis in the treatment with acetylcholinesterase inhibitors, it is aimed to reduce the amount of acetylcholine lost by inhibiting acetylcholinesterase, the enzyme that breaks down acetylcholine (Selekler, 2010; Berk, Sabbagh, 2012). Acetylcholine is not only common in the central nervous system (CNS). It is the main neurotransmitter used by the parasympathetic nervous system, lowering the heart rate and stimulating the gastrointestinal tract and bladder. Acetylcholinesterase also plays a role here, so inhibition of

***Correspondence:** A. Bozkir. Department of Pharmaceutical Technology. Faculty of Pharmacy. Ankara University. Ankara, Turkey. Phone: +90-312-203 30 00. E-mail: bozkir@pharmacy.ankara.edu.tr. ORCID: https://orcid.org/0000-0002-2782-3280. G. R. TOPAL - https://orcid.org/0000-0002-7715-6383. B. KÜÇÜKTÜRKMEN - https://orcid.org/0000-0001-7026-8932. U. C. ÖZ - https://orcid.org/0000-0001-5225-748X. E. ÖZKAN - https://orcid.org/0000-0001-9461-2339. F. BAKAR-ATES - https://orcid.org/0000-0003-2809-8946

acetylcholinesterase causes parasympathetic activation. It causes some side effects such as nausea, vomiting, and diarrhea. Another important side effect is an increased risk of bradycardia and fainting (Chinthapalli, 2017).

One of the most important problems regarding the treatment of AD is the presence of the blood-brain barrier (BBB), a highly selective diffusion barrier that protects the brain from toxins and other compounds from the blood (Zenaro, Piacentino, Constantin, 2017). To overcome the BBB, many drug delivery systems such as liposomes, solid lipid nanoparticles (SLNs), polymeric nanoparticles, micelles, nanoemulsions, and nanogels have been developed (Topal *et al*., 2021; Küçüktürkmen, Bozkır, 2018; Fonseca-Santos, Gremiao, Chorilli, 2015). To cross the BBB readily, solid lipid nanoparticles (SLNs) can be used as a drug delivery system for active substances due to their similarity to the lipophilic BBB.

SLNs are an alternative carrier system to emulsions, liposomes, and polymeric nanoparticles (Naseri, Valizadeh, Zakeri-Milani, 2015). They are submicron particles with the advantages of these delivery systems and are generally made up of biocompatible lipids or lipid mixtures and stabilized with emulsifying agents in an aqueous dispersion (Jain, Thareja, 2019; Sarangi, Padhi, 2016). As a solid lipid matrix component, tripalmitin lipid provides higher loading efficiency at low rates due to its high crystalline structure (Behbahani *et al*., 2017). Tripalmitin SLNs have recently been used to deliver anticancer etoposide across the blood-brain barrier (BBB) (Kuo, Chao, 2016). In the preparation of SLN formulations, hydrophilic surfactants such as Tween 80 and Pluronic F68 have been proven to produce higher emulsification efficiency and smaller particles compared to lipophilic surfactants. Combinations of hydrophilic and lipophilic surfactants were found to improve stability as well as entrapment efficiency (Wu, Sweeney, Dudhipala, 2021).

Donepezil HCl is the most widely used acetylcholinesterase inhibitor in the treatment of disease today. It has been discovered that it improves cognitive aspects in Alzheimer's patients ranging from mild to severe (Korabecny *et al*., 2014). Studies have shown that Donepezil HCl reduces the neural toxicity of amyloid-beta (Aβ) and affects the amyloid precursor protein (APP) process, in addition to its cholinergic effects on the disease. It acts by interfering with the exposure process of the toxic N-terminal region of APP in the formation of Aβ and is thought to prevent the onset of neurodegeneration (Jacobson, Sabbagh, 2008). Donepezil HCl was usually used alone as a standard drug and positive control in SLN formulation studies developed for the treatment of Alzheimer's disease (Yusuf *et al*., 2013; Rishitha, Muthuraman, 2018; Sathya, Shanmuganathan, Devi, 2020). There are few lipid-based formulations of Donepezil HCl that were reported to utilize liposomes (Al Asmari *et al*., 2016; Zhang *et al*., 2019), cubosomes (Patil *et al*., 2019), and SLNs (Yasir *et al*., 2018). In a study, SLN formulations containing donepezil were developed for nose-to-brain delivery, and it was stated that the concentration in the brain increased compared to the donepezil solution (Yasir *et al*., 2018).

While developing in vitro models for AD, cancer cell lines, primary neuronal cells, or neural stem cells are frequently used (Deli *et al*., 2005). In cellular studies for Alzheimer's disease, both the human neuroblastoma cell line (SH-SY5Y) and the mouse brain microvascular endothelial cell line (bEnd.3) have been evaluated together (Ali *et al*., 2021; Zeng *et al*., 2022). Since the SH-SY5Y cell line is thought to mimic neurons in physiological and pathological conditions, it is frequently used in in vitro models for AD and in neurodegeneration mechanism studies (Calan *et al*., 2016; Gao *et al*., 2017). The bEnd.3 cell line is also frequently used in AD cell culture studies because of its paracellular barrier properties (Watanabe *et al*., 2013).

In this study, it was aimed to prepare Donepezil HCl-loaded solid lipid nanoparticles to enable Donepezil HCl to easily cross the BBB by using loaded SLNs and to reduce side effects by using fewer doses. When the particle size is under 200 nm, particles can cross the BBB easily, so we aimed to obtain about 200 nm SLNs. We examined many formulation parameters to optimize the process of production and achieve the best formulation. After the preparation of SLNs; particle size, polydispersity index, zeta potential, and encapsulation efficiency studies were performed. Also study releases, TEM analyses and cytotoxicity tests were carried out on selected formulation.

MATERIAL AND METHODS

Material

Donepezil HCl was kindly gifted by Deva Holding Corp. (İstanbul, Turkey); Tripalmitin was also a gift from IOI Oleochemical. Tween® 80 and Pluronic F68 were the products of Merck (Germany). Polyvinyl alcohol (PVA) (Mw 30000-70000), Arlacel®-C (sorbitan sesquioleate), and dichloromethane (DCM) were purchased from Sigma-Aldrich (Germany). Amicon Ultra-4 centrifuge tubes were from Millipore, USA. All other chemicals used were of analytical grade.

Preparation of SLNs

SLNs were obtained by using a homogenization/ sonication method. The effects of various formulation parameters on the physicochemical properties of SLNs like type of surfactant, concentration of Donepezil HCl, surfactant, tripalmitin homogenization time and speed, and sonication time were examined. As a sum up, the lipid tripalmitin was heated to 75-80 °C, and the active substance, Donepezil HCl was dispersed in the lipid phase. The aqueous phase was heated to 75 - 80 °C. Surfactant was added according to its properties to the oil or aqueous phase. The water phase was added to the oil phase and then mixed with the high-speed mixer (Ultra-Turrax T-25, IKA, Germany). Afterwards, coarse emulsion was sonicated (Sonopuls, Bandelin, Germany) and then allowed cool to room temperature. The formulations were finally centrifuged at 4000× g for 20 min at 4 °C for three times using Amicon Ultra-4 centrifuge tubes, and the pellets were collected (Figure 1). The formulations are given in Table I.

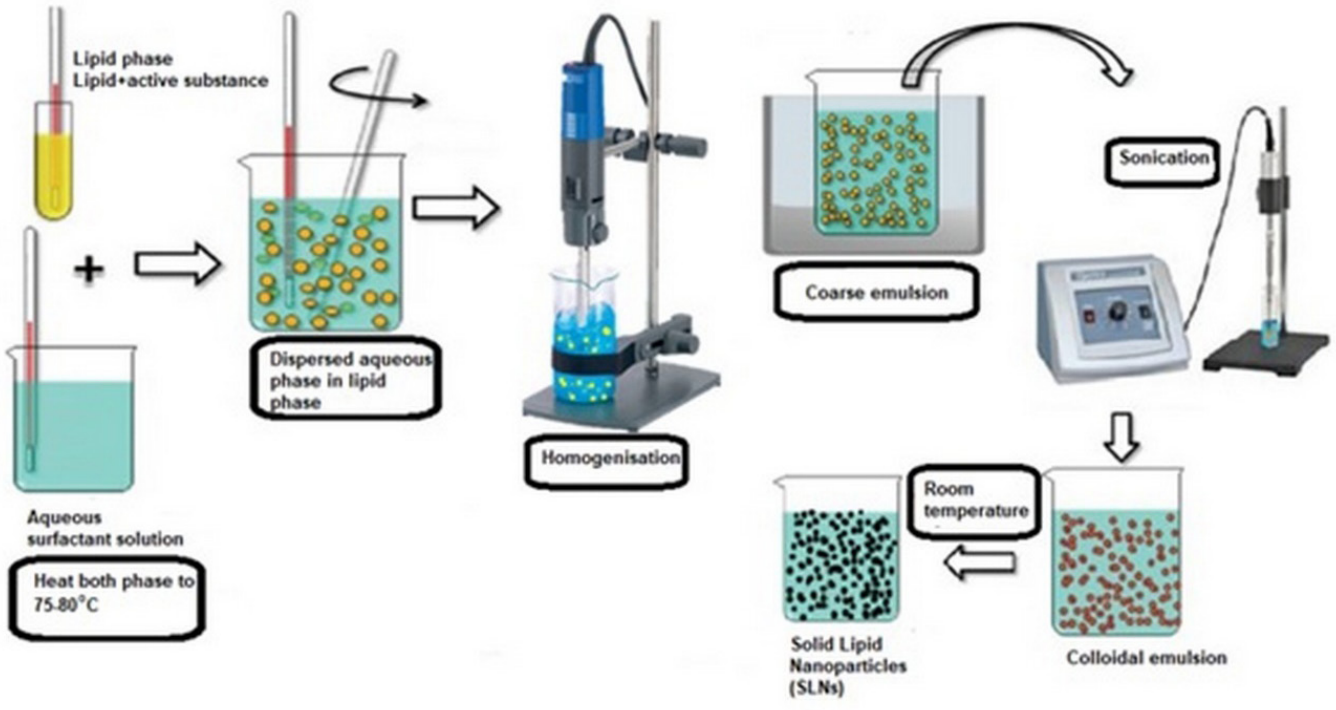

FIGURE 1 - Preparation of SLNs with homogenization/sonication method.

Particle size, polydispersity index and zeta potential

For measurement of the particle size distribution and zeta potential of SLNs, the dispersion of particles was diluted with ultrapure water at 200 µg/ml concentration and all measurements were done at 25°C using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) and each sample was analyzed in triplicate.

HPLC analysis of Donepezil HCl

Donepezil HCl concentrations were measured by HPLC (Agilent 1260 Infinity, Agilent Tech., Germany). C18 column (ACE, UK; 4.6×250 mm) was used at 37 °C. The mobile phase was a mixture of methanol and bidistilled water (85:15, v/v). The flow rate was 0.5 ml/min and the injection volume was 20 µl. The eluent was monitored at 270 nm and retention time was 4.5 min.

Encapsulation efficiency

The encapsulated amount of Donepezil HCl in the SLNs was determined using the indirect method from the supernatants. After centrifugation of SLNs using Amicon Ultra-4 centrifuge tubes, the active substance in the supernatant was detected by HPLC immediately. The encapsulation efficiency (EE%) was calculated by using the following equation:

EE (%) for Donepezil HCl=(Amount of total drug-Amount of loaded drug)/(Amount of total drug)×100

TEM analysis

The morphology of optimal SLN formulation was investigated using a transmission electron microscope (TEM) (Feı - Tecnai G2 F20 S-Twin). A drop of the SLN formulation was placed on 200 mesh copper grids, and the excess of SLN was taken with filter paper. The sample was stained with a 2% uranyl acetate solution for 2 minutes, and it was dried under vacuum, and visualized at 120 kV.

TABLE I - Optimization of various formulation parameters for the preparation of SLNs

Code	Lipid	Addition of API	Lipid concentration (% w/v)	Surfactant in lipid phase	Surfactant concentration (% w/v)	Volume of aqueous phase (ml)	Type of surfactant in aqueous phase	Surfactant in aqueous phase (mg)	Homogenisation speed (rpm)	Amount of API (mg)	Sonication time (min)
F1	Tripalmitin	Powder	1	--	--	10	Tween 80	100	9600	10	10
F2	Tripalmitin	Powder	2	--	--	10	Tween 80	100	9600	10	10
F3	Tripalmitin	Powder	4	--	--	10	Tween 80	100	9600	10	10
F4	Tripalmitin	Powder	8	--	--	10	Tween 80	100	9600	10	10
F5	Tripalmitin	Powder	1	--	--	3	Tween 80	100	9600	10	10
F6	Tripalmitin	Powder	1	--	--	50	Tween 80	100	9600	10	10
F7	Tripalmitin	Powder	1	--	--	10	Tween 80	100	9600	2	10
F8	Tripalmitin	Powder	1	--	--	10	Tween 80	100	9600	5	10
F9	Tripalmitin	Glycerine solution	1	--	--	10	Tween 80	100	9600	10	10
F10	Tripalmitin	ACN:Dioxan	1	--	--	10	Tween 80	100	9600	10	10
F11	Tripalmitin -1/4lecithin	Powder	1	--	--	10	Tween 80	100	9600	10	10
F12	Tripalmitin -1/2lecithin	Powder	1	--	--	10	Tween 80	100	9600	10	10

TABLE I - Optimization of various formulation parameters for the preparation of SLNs

Code	Lipid	Addition of API	Lipid concentration (% w/v)	Surfactant in lipid phase	Surfactant concentration (% w/v)	Volume of aqueous phase (ml)	Type of surfactant in aqueous phase	Surfactant in aqueous phase (mg)	Homogenisation speed (rpm)	Amount of API (mg)	Sonication time (min)
F13	Tripalmitin	Powder	1	--	--	10	Pluronic F68	100	9600	10	10
F14	Tripalmitin	Powder	1	--	--	10	PVA	100	9600	10	10
F15	Tripalmitin	Powder	1	Arlacel-C	10	10	Tween 80	100	9600	10	10
F16	Tripalmitin	Powder	1	Arlacel-C	10	10	PVA	100	9600	10	10
F17	Tripalmitin	Powder	1	Arlacel-C	10	10	Pluronic F68	100	9600	10	10
F18	Tripalmitin	Powder	1	--	10	10	Tween 80	50	9600	10	10
F19	Tripalmitin	Powder	1	--	10	10	Tween 80	150	9600	10	10
F20	Tripalmitin	Powder	1	--	10	10	Tween 80	200	9600	10	10
F21	Tripalmitin	Powder	1	--	10	10	Tween 80	250	9600	10	10
F22	Tripalmitin	Powder	1	--	10	10	Tween 80	300	9600	10	10
F23	Tripalmitin	Powder	1	--	10	10	Tween 80	150	15000	10	10
F24	Tripalmitin	Powder	1	--	10	10	Tween 80	150	20000	10	10
F25	Tripalmitin	Powder	1	--	10	10	Tween 80	150	20000	10	1
F26	Tripalmitin	Powder	1	--	10	10	Tween 80	150	20000	10	2
F27	Tripalmitin	Powder	1	--	10	10	Tween 80	150	20000	10	5

In vitro release study

The in vitro release study was carried out using the dialysis bag method. SLN samples (1 mL) in dissolution medium (pH 7.4, PBS containing 0.1% Tween 80) were put into dialysis bags (12Kda MWCO) and placed into 50 mL of dissolution medium, then shaken horizontally in the water bath (GFL 1083, GFL mbH, Burgwedel, Germany) at 50 rpm at 37 ◦C. 1 mL of sample was taken, and 1 mL fresh dissolution medium was added at certain time intervals (0.25, 0.5, 1, 2, 4, 6, 8, 12 and 24 h). The samples were filtered and analyzed using HPLC. All experiments were performed in triplicate for each of the samples.

Cyctotoxicity of SLNs

SH-SY5Y neuroblastoma cell line and bEND.3 cell line were used to examine the effects of formulations on cell viability. The effect of formulations on cell viability was investigated through the MTT assay. For SH-SY5Y, 2.5 x 104 cells/mL (180 µL volume) and for bEND.3.5 x 104 cells/mL (180 µL volume) were seeded on 96-well plates. After 24 hours, the samples were applied to the cells with fresh medium in 4 repetitions. After 24 hours, the plates were aspirated, and 20 µl of 5 mg/ml MTT agent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide) and 180 µl DMEM-F12 medium were applied. After 2 hours, the plates were aspirated again, 100 µl of DMSO was added, and the spectrophotometric measurement was taken at 540 nm.

Statistical analysis

All the data were expressed as a mean±standard deviation. A Student's test was used to compare differences between groups. P values less than 0.05 were considered statistically significant. Each experiment and analysis was performed as three replications.

RESULTS AND DISCUSSION

Effect of lipid concentration on the physicochemical properties of SLNs

Since the change in tripalmitin concentration affects the solubility of the active substance, it is important for encapsulation efficiency; however, the particle size and distribution must be considered when altering the concentration, because the change in lipid concentration generally results in an increase in particle size.

As presented in Table II, the particle size and PDI increased with increasing lipid concentration from 1% (w/v) to 2% (w/v) and 4% (w/v). The particles grew from nano to micron size with increasing lipid concentration. There was a significant difference between formulations in zeta potential value ($p<0.05$). The results were better at 1% (w/v) lipid concentration. In similar studies, particle size and PDI values increased with increasing lipid concentration (Martins *et al*., 2012; Pooja *et al*., 2016). The encapsulation efficiency has not been investigated for F2 and F3 since the particles were microns in size.

TABLE II - Effect of lipid concentrations on SLNs

Code	Lipid concentration (% w/v)	Particle size (nm±SD)	PDI (Mean ±SD)	Zeta (mV±SD)	EE (%±SD)
F1	1	100.4±0.50	0.13±0.01	-18.3±0.36	85.17±1.21
F2	2	1190.0±0.17	1.00±0.00	-22.3±0.21	*
F3	4	8612.5±0.05	1.00±0.00	-17.3±0.62	*
F4	8	No emulsion formation			

*Since these particles were not nanometers in size, encapsulation efficiencies were not determined.

Volume of aqueous phase

The volume of the aqueous phase used in the outer phase can affect the efficiency of mixing and sonication. There was a significant difference between all results for the formulations coded 10 ml F1 and 50 ml F6 ($p<0.05$) (Table III). When the formulations were compared, F1 had better results. This situation may have been caused by insufficient mixing speed and sonication time due to the larger aqueous phase volume. As a result, the particle size and distribution increased and the zeta potential decreased. When the F5 with less water phase volume is compared with the F1, it is seen that the particle size increases and the zeta potential and encapsulation efficiency decrease in the F5 coded formula ($p<0.05$). This may be related to the sonication power and time applied during the formation of the formulation (Behbahani *et al*., 2017; Ibrahim, Al-Omrani, Yassin, 2013).

TABLE III - Effect of aqueous phase volume on the physicochemical properties of SLNs

Code	Volume of aqueous phase (mL)	Particle size (nm±SD)	PDI (Mean±SD)	Zeta (mV±SD)	EE (%±SD)
F1	10	100.4±0.50	0.13±0.01	-18.3±0.36	85.17±1.21
F5	3	247.0±0.35	0.79±1.45	-8.9±0.45	83.40±3.29
F6	50	121.0±0.09	0.33±0.02	-11.3±0.22	86.20±2.85

Effect of the Donepezil HCl concentration

As the amount of Donepezil HCl increased, the particle size and distribution increased (Table IV). This may be due to the incorporation of the active ingredient into the nanoparticle core, which leads to an increase in particle size and distribution (Behbahani *et al.*, 2017). In formulations coded as F1, containing 10 mg of active ingredient, F9 containing 2 mg of active ingredient, and F8 containing 5 mg of active ingredient, the active ingredient was directly added to the lipid phase as solid powder. There was no significant difference in particle size or PDI between them ($p>0.05$). In this case, it can be said that the active ingredient/lipid ratio used in F1 has no effect on changing the particle size, and this amount can be tolerated by the lipid. The difference between zeta potentials was found to be significant ($p<0.05$) and the F1 coded formulation exhibited better colloidal stability. While there was a significant difference between F1 and F9 in terms of encapsulation efficiency ($p>0.05$); there was no significant difference between F1 and F8. The encapsulation efficiency is directly proportional to the Donepezil HCl concentration; therefore, when increased from 2 mg to 5 and 10 mg, the loading efficiency also increased; however, each lipid has a loading capacity, and once this maximum is reached, the encapsulation efficiency will no longer increase; this causes the excess active ingredient not to be loaded and remains in the aqueous phase (Das, Chaudhury, 2011).

TABLE IV - Effect of Donepezil HCl amount on SLNs

Code	Addition of AS	Amount of AS (mg)	Particle size (nm±SD)	PDI (Mean±SD)	Zeta (mV±SD)	EE (%±SD)
F1	Powder	10	100.4±0.50	0.13±0.01	-18.3±0.36	85.17±1.21
F7	Powder	2	101.3±0.53	0.14±0.01	-12.3±0.23	80.02±1.21
F8	Powder	5	102.4±0.23	0.20±0.03	-15.7±0.02	85.60±1.10
F9	Glycerine solution	2	62.8±0.22	0.23±0.20	-4.5±0.03	99.90±0.90
F10	ACN:Dioxane	2	58.0±0.45	0.22±0.01	-3.2±0.01	82.55±0.10

ACN: Acetonitrile

Effect of lecithin

Lecithin is added as a co-surfactant in SLNs and acts as an internal phase surfactant; It has a positive effect on particle size and stability (Pooja *et al.*, 2016). When the F1 and F11, F12 formulations are compared, it can be seen that the particle size decreases significantly, and the encapsulation efficiency increases in the lecithin-containing F11 and F12 formulations ($p<0.05$) (Table V). PDI and zeta potential were negatively affected by lecithin ($p<0.05$). Similar results were obtained in previous studies (Liu *et al.*, 2007). In the Precirol SLNs which contain lecithin and without lecithin, particle size decreased (from 42.7 nm to 37.33 nm) and increased the encapsulation efficiency (82.62% to 95.59%) significantly with lecithin. Due to the amphoteric nature of lecithin, the solubility of the active ingredient in the lipid phase may have increased; so this will positively affect the encapsulation efficiency.

TABLE V - Effect of lecithin on SLNs

Code	Lipid	Lipid concentration (% w/v)	Particle size (nm±SD)	PDI (Mean±SD)	Zeta (mV±SD)	EE (%±SD)
F1	Tripalmitin	1	100.4±0.50	0.13±0.01	-18.3±0.36	85.17±1.21
F11	Tripalmitin	1	67.8±0.56	0.24±0.10	-7.4±0.02	99.89±0.30
	Lecithin	0.25				
F12	Tripalmitin	1	72.4±0.15	0.35±0.05	-5.4±0.30	99.89±0.36
	Lecithin	0.5				

Effect of the type of surfactant

SLNs are colloidal systems in which solid lipids are stabilized and formed in the aqueous phase using surfactants. Therefore, the choice of surfactant is very important (Gupta *et al.*, 2017). For this purpose, SLNs were evaluated by adding different surfactants to the water phase and lipid phase. The results are given in Table VI.

We examined Arlacel-C in the lipid phase and Tween 80, Pluronic F68 and PVA in the aqueous phase. When the effect of surfactant type is examined in the literature, the general trend is that polysorbates (Tween 80) form SLNs with smaller particle sizes than poloxamers (Pluronic F68) (Martins *et al.*, 2012).

In F13 and F17 coded formulas prepared with Pluronic F68 (Poloxamer 188), the increase in particle size can be seen markedly compared to other formulations ($p<0.05$). When the encapsulation efficiencies of F1 and F13 coded formulas were compared, there was a significant increase ($p<0.05$). It was thought that as the particle size got bigger, the active substance that loaded into particle increased.

Although the particle size was acceptable in the F14 coded formula prepared with PVA, the PDI was higher than F1 ($p<0.05$) and a decrease in encapsulation efficiency was observed ($p<0.05$). In the particles prepared with PVA, PVA is absorbed around the emulsion droplets during the homogenization at the particle formation stage, and spontaneous droplet formation ends with particle formation in the micron range (Hu *et al.*, 2002).

An increase in particle size and a decrease in encapsulation efficiency were observed in formulations with Arlacel-C added to the lipid phase ($p<0.05$). Arlacel-C is a hydrophilic surfactant with an HLB value of 11 used for the encapsulation of active ingredients (Küçüktürkmen, Öz, Bozkır, 2017). The addition of this surfactant in the formulation may have increased the solubility of the active substance in the aqueous phase and therefore may have allowed the active substance to escape into the aqueous phase.

TABLE VI - Effect of surfactant type on SLNs

Code	Type of surfactant for lipid phase	Type of surfactant for aqueous phase	Particle size (nm±SD)	PDI (Mean±SD)	Zeta (mV±SD)	EE (%±SD)
F1	--	Tween 80	100.4±0.50	0.13±0.01	-18.3±0.36	85.17±1.21
F13	--	Pluronic F68	204.3±0.36	0.36±0.04	-11.0±0.20	95.78±0.08
F14	--	PVA*	120.6±0.12	0.45±0.33	-16.5±0.03	65.17±1.33

TABLE VI - Effect of surfactant type on SLNs

Code	Type of surfactant for lipid phase	Type of surfactant for aqueous phase	Particle size (nm±SD)	PDI (Mean±SD)	Zeta (mV±SD)	EE (%±SD)
F15	Arlacel-C	Tween 80	156.8±0.22	0.22±1.56	-26.8±0.10	29.50±0.69
F16	Arlacel-C	PVA	250.4±0.23	0.18±0.25	-12.6±0.36	8.24±0.03
F17	Arlacel-C	Pluronic F68	303.5±1.37	0.18±0.66	-17.2±0.02	24.33±0.54

*PVA: Polyvinyl alcohol

Amount of Tween 80 as surfactant

Surfactant concentration is highly effective for particle size and distribution. The particle size and distribution of formulations decreased with the increase in the amount of surfactant ($p<0.05$) (Table VII). Similar results have been obtained by other researchers (Liu *et al.*, 2007; Hu, Tang, Cui, 2004). These results show that production with a high concentration of Tween 80 led to smaller and more stable particles, which may be due to the presence of sufficient surfactant to stabilize the nanodroplets. F1 and F19 formulations, which contain 100 mg and 150 mg Tween 80 respectively, have encapsulation efficiencies over 85%. A sufficient amount of surfactant in the formulations may have helped the active substance remain in the lipid particle or on the particle surface.

TABLE VII - Effect of surfactant's amount on SLNs

Code	Amount of Tween 80 (mg)	Particle size (nm±SD)	PDI (Mean±SD)	Zeta (mV±SD)	EE (%±SD)
F1	100	100.4±0.50	0.13±0.01	-18.3±0.36	85.17±1.21
F18	50	889.5±5.23	0.80±0.19	-3.5± 0.01	*
F19	150	96.3±2.33	0.25±0.50	-18.3±0.01	86.34±0.02
F20	200	55.6±0.78	0.22±2.14	-17.0±0.30	82.37±0.03
F21	250	35.3±0.89	0.26±0.98	-4.5±0.36	85.69 ±0.38
F22	300	34.7±0.30	0.27±0.10	-2.3±0.27	93.65 ±0.02

*Since these particles were not nanometers in size, encapsulation efficiencies were not determined.

Homogenization speed

The homogenization speed has an effect on the formation of SLNs and, accordingly, on particle size and distribution and the zeta potential. Decrease of the homogenization speed may lead the oil droplets not breaking sufficiently during emulsification process that can be resulted big particle size and large particle distribution in the formulation (Siddiqui *et al.*, 2013).

Smaller particles were obtained at 20000 rpm (Table VIII). The fact that the PDI value is lower than the other formulations indicates that a more monodisperse system is obtained. Considering the significant increase in encapsulation efficiency, it was found appropriate to continue the study with the F24 coded formula.

TABLE VIII - Effect of homogenization speed on SLNs

Code	Homogenization speed (rpm)	Particle size (nm±SD)	PDI (Mean±SD)	Zeta (mV±SD)	EE (%±SD)
F19	9600	96.3±2.33	0.25±0.50	-18.3±0.01	86.34±0.02
F23	15000	100.4±0.32	0.39±0.23	-23.3±0.02	87.29±0.01
F24	20000	87.2±0.11	0.22±0.02	-17.0±0.12	93.84±0.01

Sonication time

Sonication time is one of the most important formulation parameters affecting particle size and distribution. In general, the longer the sonication time results the smaller the particle size and distribution get. Sonication breaks the coarse emulsion droplets and turns the emulsion into nano-emulsion, which in this case leads to a decrease in particle size and dispersion (Das *et al.*, 2011). In our study, the particle size and PDI decreased with the prolongation of the sonication time ($p<0.05$) (Table IX). When the results were examined, it was thought that the study should be continued with a 10-minute sonication time.

TABLE IX - Effect of sonication time on SLNs

Code	Sonication time (min)	Particle size (nm±SD)	PDI (Mean±SD)	Zeta (mV±SD)	EE (%±SD)
F24	10	87.2±0.11	0.22±0.02	-17.0±0.12	93.84±0.01
F25	1	130.8±0.62	0.24±0.01	-16.6±0.49	85.20±0.56
F26	2	112.3±1.22	0.26±0.01	-15.6±0.98	86.70±0.06
F27	5	105.5±0.50	0.25±0.01	-12.3±0.65	86.30±0.03

TEM analyses

TEM analyses were carried out to examine the morphological properties of nanoparticles. When the TEM images of SLNs were examined, it was observed that spherical and smooth surface nanoparticles were obtained. TEM image of F24 is given in Figure 2.

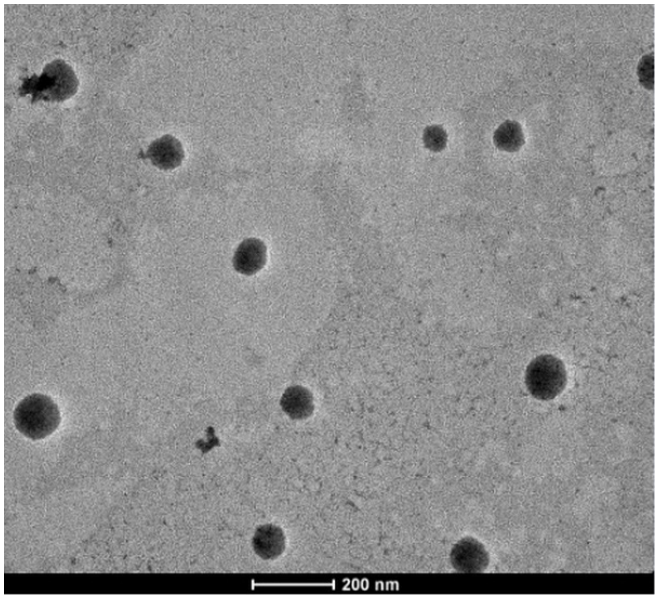

FIGURE 2 - TEM image of F24.

In vitro release study

The in vitro release of Donepezil HCl and the F24 coded formulation, which was Donepezil HCl loaded SLNs, were compared (Figure 3). The release profile of Donepezil HCl from F24 showed an initial burst release followed by a slower release profile. 20% of Donepezil HCl from F24 and 66% of the free drug were released in the first hour. The reason for the burst effect seen in the first hours can be explained by the free active substance on the nanoparticle surface (D'Souza, 2014). Donepezil HCl on the particle separated from the particle surface quickly, so it seemed like a burst release. At the end of the 24th hour, the free drug was released completely, and F24 reached a release profile aprroximately 70% of the total drug. F24 coded formulation clearly showed a slower release profile than the free drug. The nanoparticle slowed the release. It can be said that the homogeneous distribution of the active substance in the lipid matrix and the release due to the diffusion of the active substance from the lipid matrix could cause the slow release profile (Singh *et al.*, 2010; Amasya *et al.*, 2021).

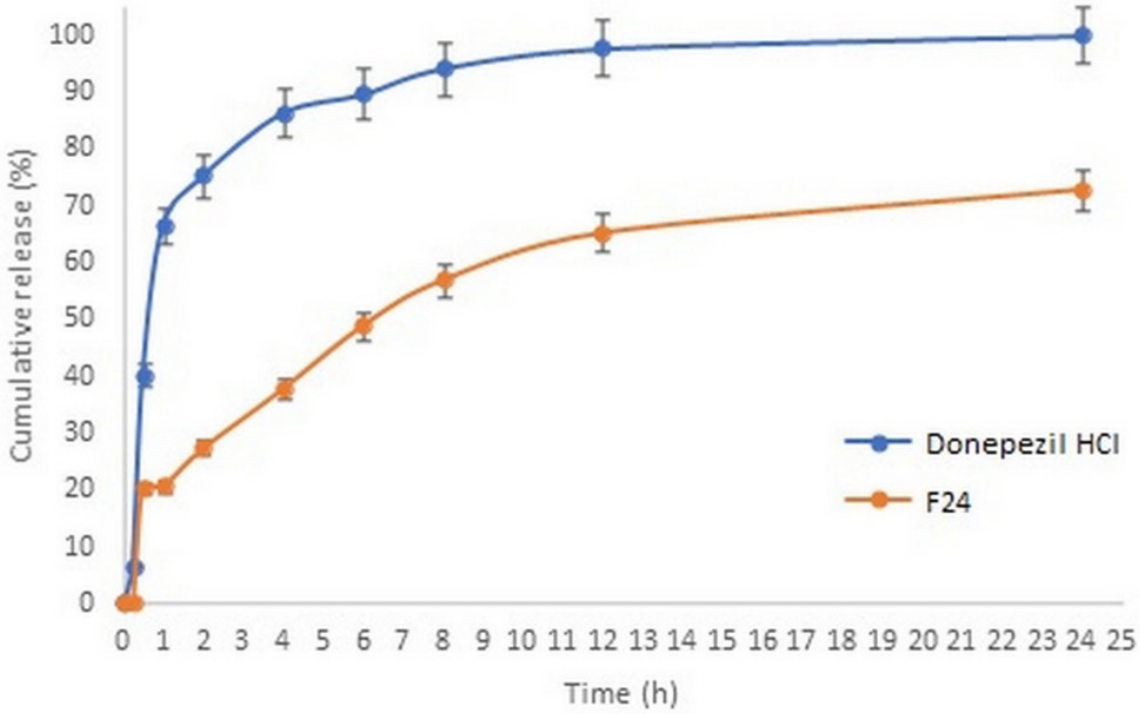

FIGURE 3 - In-vitro release study for pure Donepezil HCl and F24 coded formulation.

Cyctotoxicity of SLNs

MTT studies on the SH-SY5Y cell line were examined, and when cell viability values obtained from various doses of Donepezil pure solution and blank formulation were compared with the control group, no significant effect was observed in 24 hours ($p>0.05$). For the F24 coded formula, a significant difference was observed at concentrations of 1 µg/mL ($p<0.05$) and 10 µg/mL ($p<0.0001$) (Figure 4).

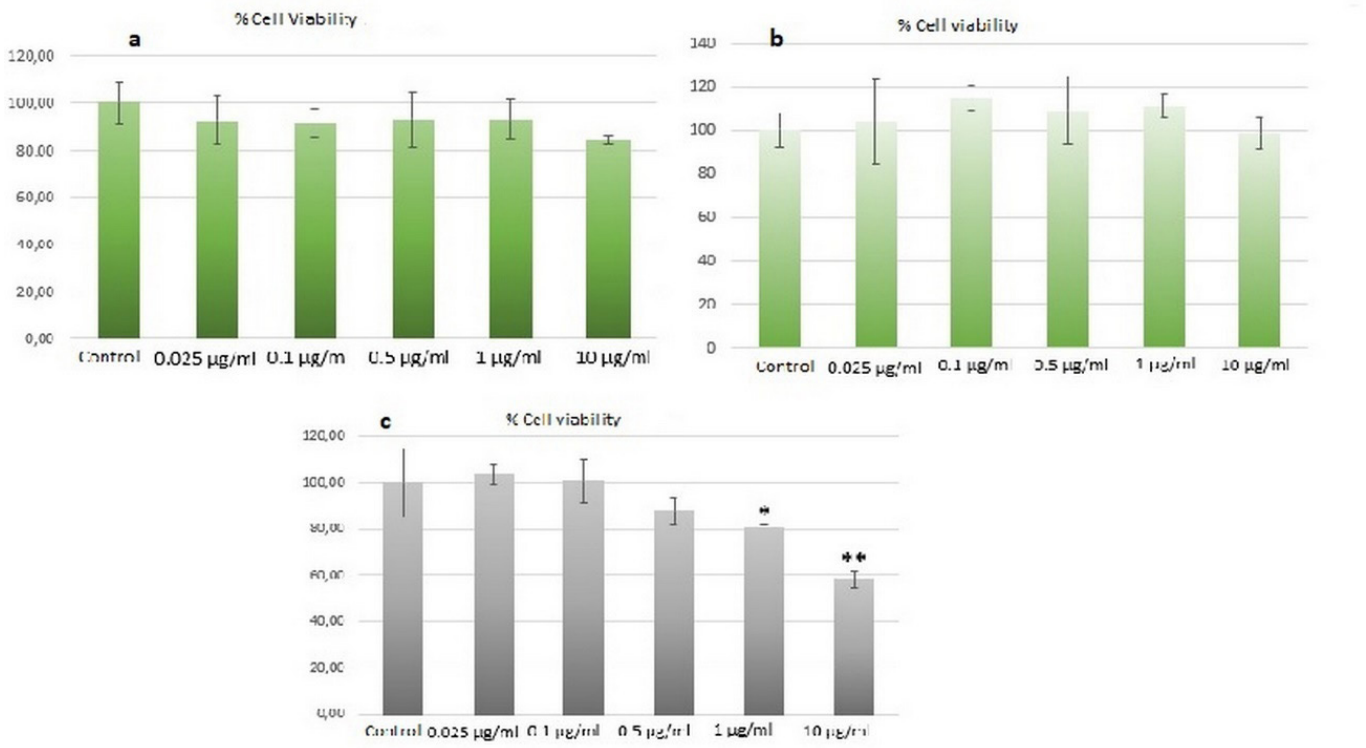

FIGURE 4 - Cell viability results of Donepezil HCl solution (a), Blank (b) and F24 formulations (c) on SH-SY5Y cell lines *$p<0.01$ **$p<0.0001$.

MTT studies on the bEnd.3 cell line were examined, and when cell viability values obtained from various doses of Donepezil pure solution were compared with the control group, a significant difference was observed at concentrations of 10 µg/mL ($p<0.05$) (Figure 5). For blank formulations, 1 and 10 µg /mL and for F24 coded formulations, 0.1 to 10 µg/mL concentrations were effective on cell viability. This may be due to the fact that SLN was rapidly internalized by entering the cell and had a toxic effect with the rapid release of the substance from the particle (Miglietta *et al.*, 2000).

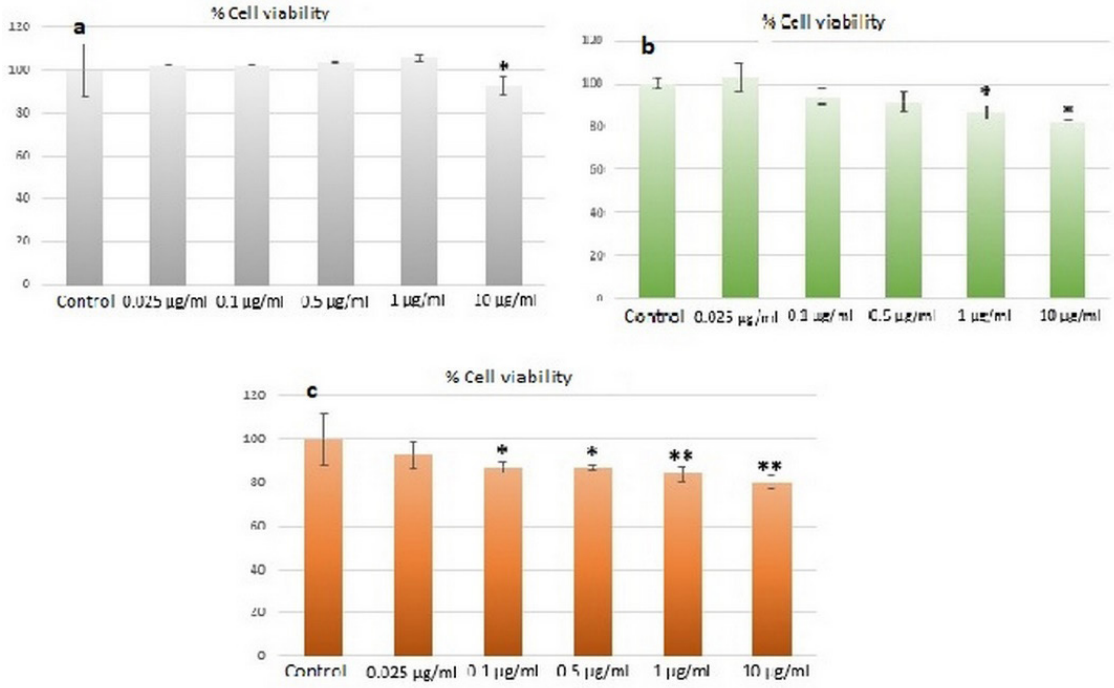

FIGURE 5 - Cell viability results of Donepezil HCl solution (a), Blank (b) and F24 formulations (c) on bEnd.3 cell lines *p<0.01 **p<0.0001.

CONCLUSION

In this study, solid lipid nanoparticles containing Donepezil HCl, the most commonly used drug in Alzheimer's disease, were designed utilizing various formulation parameters. SLNs consist of biocompatible lipids and lipid mixtures, so they are an attractive carrier system, especially for lipophilic BBB, because of their lipid formation.

We compared the different formulation parameters like lipid concentration, volume of the aqueous phase, concentration of the active substance, and different types and concentrations of surfactants. The highest lipid concentration resulted in larger particle sizes and a wider range. The volume of the aqueous phase affected the homogenization and the sonication impact during formation of SLNs, causing particle sizes to change. The addition to the formulation and concentration of Donepezil HCl were effective on particle size and EE markedly. Different types and concentrations of surfactants have resulted in incredible changes in particle sizes. Homogenization speed and sonication time were effective on particle size and distribution. Optimized SLN formulation F24 was obtained with a 20000 rpm homogenization speed and 10 minutes of sonication time. The optimized formulation displayed a particle size of 87.2±0.11 nm; a PDI of 0.22±0.02; encapsulation efficiency of 93.84%±0.01 and a zeta potential of -17.0±0.12 mV. In the release study, at the end of the 24th hour, Donepezil HCl was released at 80% of total drug when compared to free drug, it was 100%. TEM analysis proved that spherical and smooth particles could be obtained. There was no significant cytotoxicity effect on SH-SY5Y on the other hand, the cytotoxicity studies on bEnd.3 cell lines showed that cell viability decreased markedly. This optimized formulation achieved a particle size of less than 200 nm which can cross the BBB easier and possesses a high encapsulation efficiency. The final formulation could be a candidate nanocarrier for Donepezil HCl delivery.

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CONFLICT OF INTEREST STATEMENT

The authors declared no conflict of interest.

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