Synthesis, Formulation, and Characterization of Gabapentin-Phosphatidylcholine Conjugate Loaded Nanostructured Lipid Carriers

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Abstract:

Epilepsy is a neurological disorder that affects about 60 million people worldwide. Currently available antiepileptic medications have a limited shelf life, and their undesirable physicochemical and pharmacokinetic characteristics restrict their use in treating epilepsy. For example, Gabapentin, a structural analog of gamma-aminobutyric acid (GABA), a BCS class III drug, has permeability issues that lead to bioavailability problems. To overcome these issues, gabapentin is chemically conjugated with phosphatidylcholine and loaded as nanostructured lipid carriers (NLCs), which can be targeted to Phospholipase A2. This enzyme is expressed more during epilepsy in the brain. In this present study, initially, docking studies were performed between phospholipase A2 and gabapentin- phosphatidylcholine conjugate (GPC), and based on the docking score, the same were conjugated. The microemulsion technique was used to prepare conjugate (GPC) loaded NLCs and evaluated for particle size, polydispersity index, zeta potential, entrapment efficiency, and release kinetic studies. The GP conjugate was shown to have a higher Gibdock score (-77.1084) than both gabapentin (-192.535) and phosphatidylcholine (-150.212). The optimized formulation of NLCs showed acceptable ranges of particle size (50.08nm), PDI (0.473), zeta potential (-1.48 mV), and entrapment efficiency (77.8%). Finally, it was understood that the drug release kinetics from the NLCs followed Higuichi's diffusion as a mode of release, and the mechanism of drug release was non-fiction diffusion. This study concludes that GP conjugate-loaded NLCs may be used to treat epilepsy as they can cross the BBB and target the phospholipase A2 enzyme, where the prodrug will cleave and release the gabapentin.

Keywords: *Epilepsy; Gabapentin-Phosphatidylcholine conjugate; docking studies; NLCs; In-vitro release kinetics*

1. Introduction

Paroxysmal manifestations of the cerebral cortex are seizures. When the excitatory and inhibitory forces within the network of cortical neurons suddenly become unbalanced, results a seizure [1]. None of the anti-epileptic medicines (AEDs) has demonstrated maximum effectiveness in treating epilepsy, and each AED has its unique combination of negative side effects. ADEs are chosen based on adverse effects, ease of administration, cost-effectiveness, and medical professionals' familiarity with the medicine [2].

When a seizure happens, phospholipase-activity A2 increases, cleaving the phosphono moiety and producing high drug concentrations locally. Before a medicine is activated, the severity of a seizure must be established. Additionally, the drug's inherent properties may influence its usefulness in clinical settings [3, 4].

The blood-brain barrier (BBB) and drug interactions with AEDs are often the two problems that the anti-epileptic medication must deal with. A) Blood-Brain Barrier (BBB) and Blood Cerebrospinal Fluid Barrier: These two barriers protect the brain from foreign contaminants (BCSFB). Lewandowsky initially wrote about the "blood-brain barrier" in 1990. The endothelium or thin obstruction and the ependymal border comprise the two distinct parts of the BBB structure. The blood-cerebrospinal fluid barrier is about 5000 times stronger than the blood-brain barrier [5].

The main difficulties in treating epilepsy stem from a drug's limited capacity to cross the blood-brain barrier and its low bioavailability. Only tiny molecules, including lipid-soluble ones and those with a molecular weight of 400–600 Da, diffuse through the BBB. Small compounds that are either water-soluble or have a molecular weight of 400–600 Da are simultaneously poorly transported via the BBB [6]. Unionized compounds with log P values around 2, molecular weights around 400 Da, and no more than 8–10 hydrogen bonds can traverse the BBB optimally [5]. A selective metabolism-driven barrier exists in addition to the physical barrier, reflecting the presence and operation of several receptors, ion channels, and protein transporters [7]. The drawbacks of AEDs are that the antiepileptic medications that are now on the market are insufficient, and their side effects make inpatient administration difficult. Antiepileptic drugs only have a symptomatic effect on seizure symptoms and do not affect epileptogenesis [8]. Antiepileptic drug use is restricted due to its adverse effects, withdrawal symptoms, harmful interactions with other medications, and high cost, especially in developing countries [9].

The second generation of lipid nanoparticles, NLCs, can be created by combining fluid and strong lipids. Researchers choose NLC over SLN because it prevents lipid recrystallization, making it the most recent innovation [10]. Since alternative lipid nanoparticles have drawbacks such as low loading capacities and drug ejection during storage, NLCs are favored over them [11]. Similar to SLN, NLCs have several advantages, including the utilization of bio-compactable lipids, regulated drug distribution from the carrier, the ability to be made on a large scale for an affordable price, and protection from the biochemical degradation of the medication [12].

This study deals with insilico docking analysis of gabapentin, phosphatidylcholine, and their conjugate to target Phospholipase A2 enzyme followed by formulation and evaluation of phosphatidylcholine conjugated gabapentin loaded nanostructured lipid carriers.

2. Materials and Methods

Gabapentin was supplied as a gift sample from Aurobindo Pharma LTD, Hyderabad, India. phosphatidylcholine, chloroform, palmitic acid, and Pluronic F68 were obtained from SISCO Research Lab. Pvt. Ltd. N, N'- Carbonyl diimidazole and oleic acid purchased from SD-Fine Chemicals Ltd.

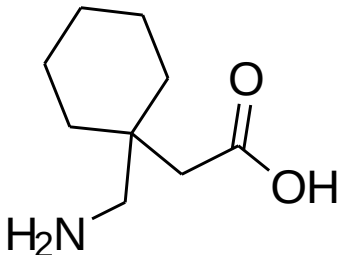
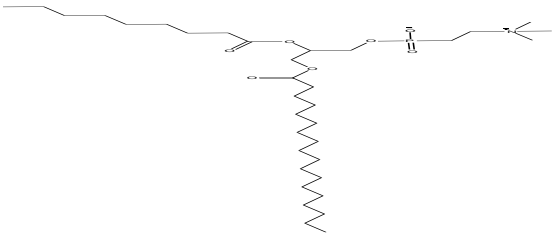
2.1 *In-silico* docking studies

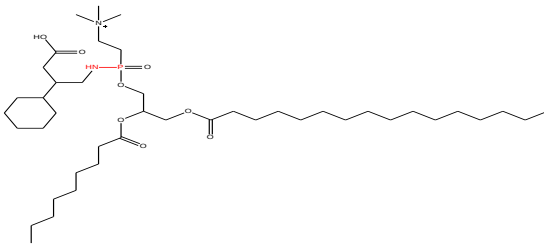
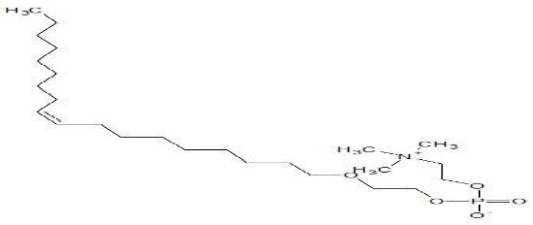
The docking analysis was done using the docking software Accelrys Discovery Studio 4.1 Client. The phospholipase A2 receptor (PDB IB- 5Y5E) has been selected from the protein data bank website.

2.1.1 Ligand preparation

The chemical structures of the gabapentin, phosphatidylcholine, gabapentin- phosphatidylcholine conjugate, and the oleyloxyethyl phosphorylcholine (inhibitor of phospholipase-2) were generated and optimized using ChemSketch (Advanced Chemistry Development, Inc. 8 King Street East, Suite 107, Toronto, Ontario, Canada M5C 1B5) software (Table-1). The ligand structures were readied utilizing Discovery Studio 4.1 through plan ligand protocol. All the tautomers and isomers of the ligand were regarded, and the ligands possessing the lowest energy has approved for docking.

Table 1: Selected candidates for *in-silico* docking studies

SI. NO	Name	Chemical structure
1	Gabapentin	
2	Phosphatidylcholine	

3	Gabapentin-Phosphatidylcholine conjugate	
4	Oleyloxyethyl phosphorylcholine	

2.1.2 Protein Preparation

The crystal structure of the investigational scaffold protein Phospholiapse A2 with inhibitor had been downloaded from the RCSB protein information bank bearing the PDB code 5Y5E (Figure 6). The protein was set up by embeddings missing atoms in inadequate buildups, demonstrating missing circle locales dependent on data, erasing substitute adaptations, expelling waters, institutionalizing particle names, and protonating titratable deposits utilizing anticipated pKas. The potential vitality, Van der Waals force, Electrostatic force, and RMS slope were checked for the protein when minimized. The protein has 123 deposits in which the edifices bound to the receptor particle, and all the hetero iotas and the superfluous water atoms were evacuated. At long last, hydrogen particles converged to the objective receptor atom utilizing Discovery Studio 4.1 Client.

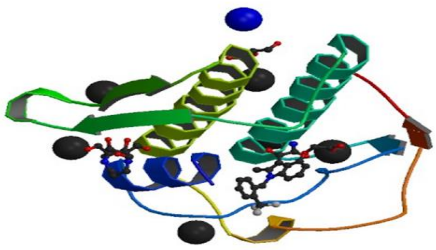


Figure 1. Three-Dimensional Structure of 5Y5E

The catalytic site prediction of phospholipase A2 with inhibitor was analyzed using Discovery Studio 4.1 Client (Figure 1). The catalytic site prediction of phospholipase A2 with inhibitor (5Y5E) was analyzed using Discovery Studio 4.1 Client. The following 74 residues were chosen as active site residues.

VAL3, GLN4, PHE5, GLY6, VAL7, MET8, ILE9, GLU10, MET 12, THR13, LYS15, SER16, ALA17, LEU18, GKN19,TYR20, GLY21, ASP22, TYR20, GLY24, CYS25, TYR26, CYS27, GLY28, ILE29, GLY28, ILE29, GLY30, GLY31, SER32, PRO35, VAL36, ASP37, GLN38, THR39, ASP40, TRP41, CYS42, CYS43, HIS44, ALA45, HIS46, ASP47, CYS48, CYS49, TYR50, GLY51, ARG52, LEU53, GLU54, GLU59, PRO60, LYS61, LEU62, GLU63, LYS64, TYR65, THR86, CYS87, GLU88, CYS89, ASP90, LYS91, ARG92, ALA93, ALA94, LEU95, CYS96, PHE97, ARG98, TYR104, TYR108, ALA109, HIS110, TYR111, ASN113, CYS116.

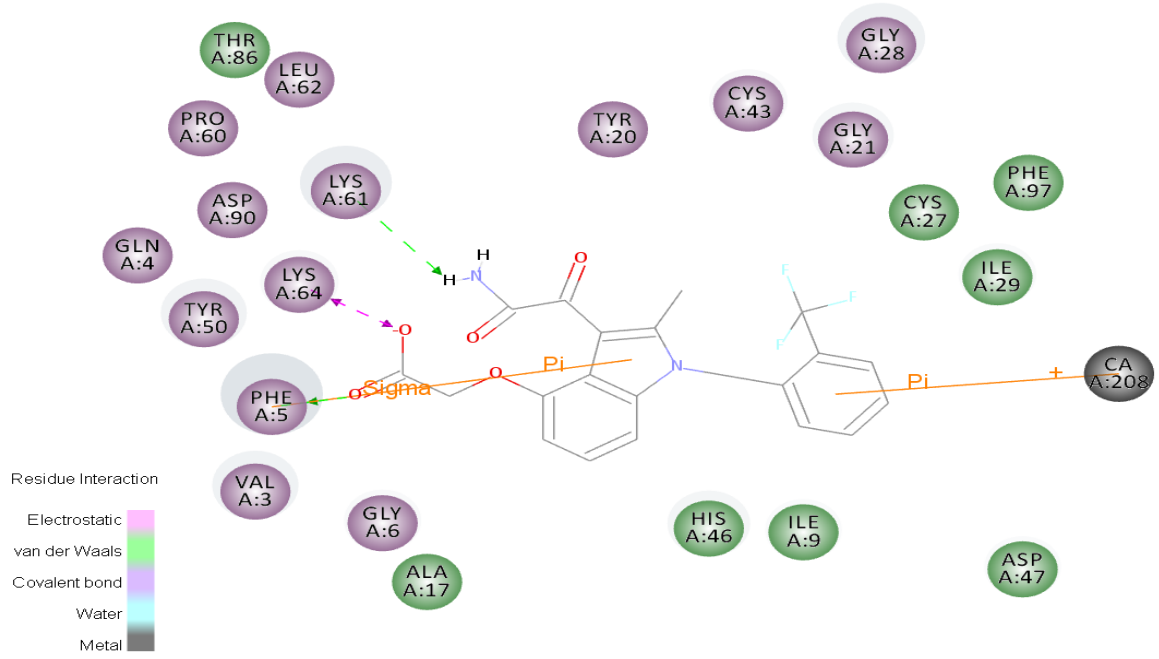


Figure 2. Active Binding Pocket using 74 residues of 5Y5E

2.1.3 Molecular Docking

A rigid-based docking program is called Libdock. Using a grid inserted into the binding site and polar and polar probes, it determines hotspots for the protein. The ligands are then aligned to create a favorable interaction using the hotspots as an additional tool. All ligand poses are ranked based on ligand scores after being reduced. From the protein data bank (PDB), the 1.8 crystal structure of phospholipase A2 associated with the native ligand (PDB ID: 5Y5E) was downloaded and imported into the Libdock working environment. The protein was made by eliminating crystal water and other heteroatoms (apart from the native ligand), adding hydrogen, protonating, ionizing, and minimizing its energy. Energy minimization was carried out using the CHARMm (Chemistry at Harvard Macromolecular Mechanics Cambridge, MA, USA) force field using the Smart minimizer method. With 2000 steps and a 0.1 RMS gradient tolerance, the minimization produced a final RMS gradient of 0.09463. The "Edit binding site" option on the toolbar for the receptor-ligand interaction was used to specify the binding site using the generated protein. The active docking sites were created using the native ligand binding locations. By using Lib dock to dock all

of the prepared ligands at the designated active site, molecular docking was completed. The 2D interactions and Libdock scores were assessed [13].

2.2 Chemical conjugation of drug and phosphatidylcholine

100 mg of phosphatidylcholine was broken down into 15 ml of chloroform. The arrangement was included drop by drop into the measuring cylinder containing 100 mg of gabapentin alongside 48 mg of N, N'-Carbonyl diimidazole. In this reaction N, N'- Carbonyl diimidazole was utilized as a coupling reagent. The response blend was on a magnetic stirrer at room temperature for 48 hours. The leftover dissolvable was expelled by utilizing a rotary evaporator. The acquired solid was washed with methylene chloride to evacuate the unreacted reagents. At last Pale yellowish shading mass was gathered and stored for further use [14]. Figure 2 speaks to the conjugation plan of gabapentin and phosphatidylcholine.

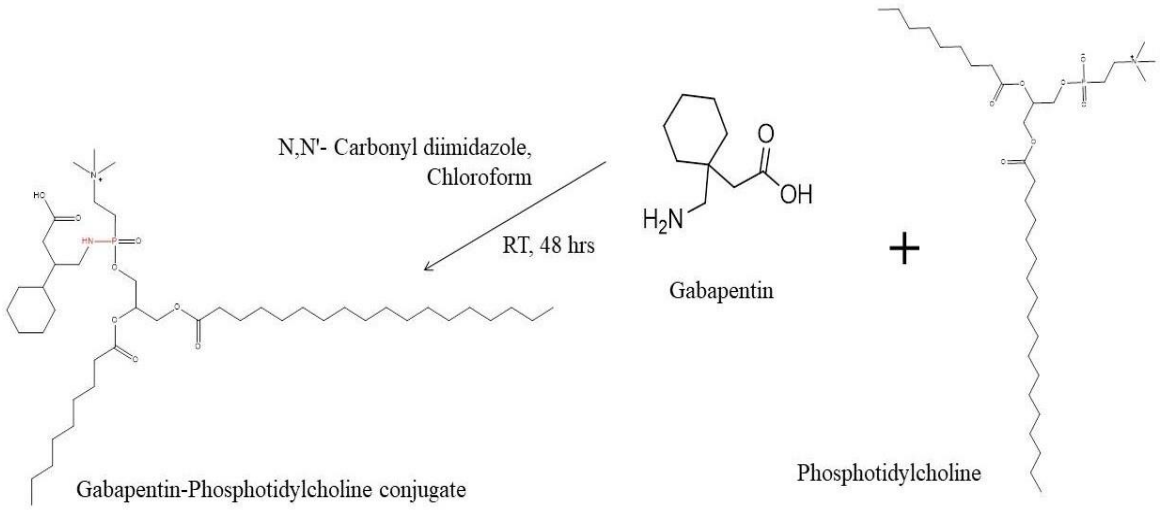


Figure 3: Conjugation plan of gabapentin and phosphatidylcholine.

2.3 Pre- formulation studies

A pre-formulation study could give essential data to plan for the adjustment required for the formulation. Every drug has regular physical properties which have been considered before the progression of pharmaceutical formulation. This property gives structure to drugs mixed with pharmaceutical excipients in production. The focus of the pre-plan considers is to develop a rich, consistent, fruitful, and safe preparation by setting up the engine rate profile, closeness with various excipients, and setting up the physicochemical parameter of the new formulation. The pre-formulation studies performed for the drug were the solubility studies and compatibility studies utilizing FTIR and DSC [15].

2.3.1 Solubility studies

The solubility characters of the gabapentin-phosphatidylcholine conjugate were resolved in different liquid lipids by including an overabundance measure of the conjugate to 10 ml of solvents in a conical flask. The conical flasks were kept at 25± 0.50^0C in an isothermal shaker for 72 hours to achieve equilibrium. The equilibrated tests were expelled from the shaker, and the supernatant was taken and examined under UV after legitimate dilution at 215 nm [16].

2.3.2 Compatibility studies: Differential scanning calorimetric studies

Differential Scanning Calorimetry, or DSC, is a thermo-consistent system in which the qualification in the proportion of warmth required to grow the temperature of a sample and reference is assessed as a temperature component. DSC may, in like manner, be used to observe progressively subtle physical changes, for example, glass propels. A Mettler TA 4000, outfitted with a DSC-30 estimating cell, was utilized for colorimetric examination. The temperature and vitality were aligned by indium as standard. Drugs and excipients were weight exclusively, and around 1 to 5 mg were put in a little aluminum pan. The pan should be sealed and placed at the point fixed. An empty pan is utilized as the reference pan. The fixings were checked at 10°C/min in the 40-300 territory. Progress temperature was characterized as following gets off the pattern in the thermogram, i.e., the beginning of the transition [17].

2.4 Formulation of NLC

Based on the solubility of the conjugate in different liquid lipids, three oils, oleic acid, castor oil, and linseed oil, were selected. Palmitic acid was selected as solid lipid and pluronic F68 as a surfactant. The NLCs were prepared using the microemulsion technique by using two ratios of each liquid lipid and solid lipid and keeping the amount of conjugate and concentration of surfactant constant. The selected lipids and surfactants were heated to 60-70^{0}C separately to prepare the lipid and aqueous phases. The melted lipid phase was added drop by drop to the aqueous phase, followed by magnetic stirring for 30 minutes. Ice-cold water is added to the hot microemulsion. The magnetic stirring was continued for 4 hours to get free-flowing NLCs.

Table 2. Various Drug Loaded NLC formulations

Liquid lipid used	Batch name	Solid Lipid (mg)	Liquid Lipid (ml)	Conjugate (mg)	Surfactant (%)
Oleic acid	**F1**	70	0.3	10mg	2%
	F2	60	0.4		
Castor oil	**F3**	70	0.3		
	F4	60	0.4		
Linseed oil	**F5**	70	0.3		
	F6	60	0.4		

2.5 Evaluation of nanostructured lipid carriers

2.5.1. Particle size, Polydispersity index (PDI), and Zeta potential

Particle size and zeta potential of the lipid nanoparticles were evaluated by corresponding photon spectroscopy using a Malvern Zetasizer Nano ZS90 (Malvern Instruments, Worcestershire, UK). All size and zeta potential estimations were done at 250 C using polystyrene cells, and disposable plain crumpled fine zeta cells [18].

Nanoparticles are usually polydisperse in nature, and the polydispersity index measures the size distribution of the nanoparticle population. The polydispersity was determined using the equation:

$$PDI= (D (0.9) - D(0.1)/ D(0.5)$$

2.5.2 Entrapment Efficiency

The NLCs were centrifuged at high rpm (10000 – 20000) for 15 – 20 min. The rpm was picked based on the atom gauge; therefore, the lesser the size, the higher the rpm required for the rotator. The accessibility of medication in the supernatant after centrifugation was managed by UV-Visible spectroscopy at 220 nm [19].

2.5.3 In-vitro drug release and kinetics studies

In in-vitro studies, the drug release from the polymeric nanoparticles was performed utilizing the dialysis membrane method. The release of GP conjugate from the NLCs was analyzed under sink conditions. 5 ml of NLCs were set in dialysis packs (MWCO 12000, HiMedia). The dialysis membrane was set in 200 mL of phosphate buffer solution (pH 7.4) and blended under a magnetic stirrer at 370^0 C$\pm 0.50^0$ C. Sample solutions were withdrawn at different intervals, and fresh PBS was added maintain the equilibrium. Test samples were analyzed using a UV Spectrometer at 215 nm for GP conjugate content.

To examine the mode of drug release from NLCs, the data were fitted into various kinetic models such as zero order, first order, Higuchi's, and Korsmayer- Peppa's models.

3. Results and Discussions

3.1 Docking analysis

The anticipated 74 active residues were utilized as catalytic residues for the three mixes utilized for the docking studies. The libdock score of standards (oleyloxyethyl phosphorylcholine), gabapentin, phosphatidylcholine, and gabapentin- phosphatidylcholine conjugate was found to be -78.6, - 192.53, -150.2, -77.12 Kcal/mol respectively. By breaking down the docking results, the gabapentin-phosphatidylcholine conjugate has a high libdock score (-77.12 Kcal/mol) while contrasting the drug, gabapentin, and phosphatidylcholine. At last, the conjugate shows the best binding communication with the Phospholipase A2. It could help distinguish proof and improve a new methodology to forestall and remedial activity against epilepsy.

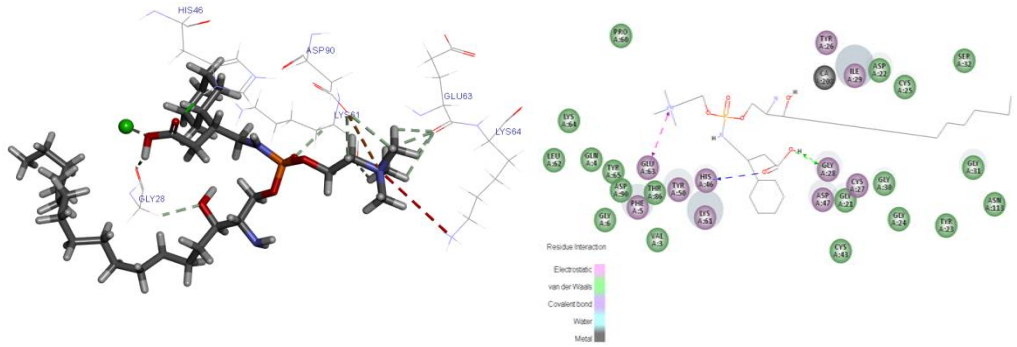


Figure 4. Docking 3D and 2D of gabapentin-phosphatidylcholine conjugate with phospholipase A2

3.2 Chemical conjugation of drug and phosphatidylcholine

After getting a pale yellowish shading mass, the same were subjected to the conformation of the formation of gabapentin- phosphatidylcholine conjugate. For this, LCMS was used to confirm there is s formation of the conjugate. The report got from LCMS is given below (Figure 5).

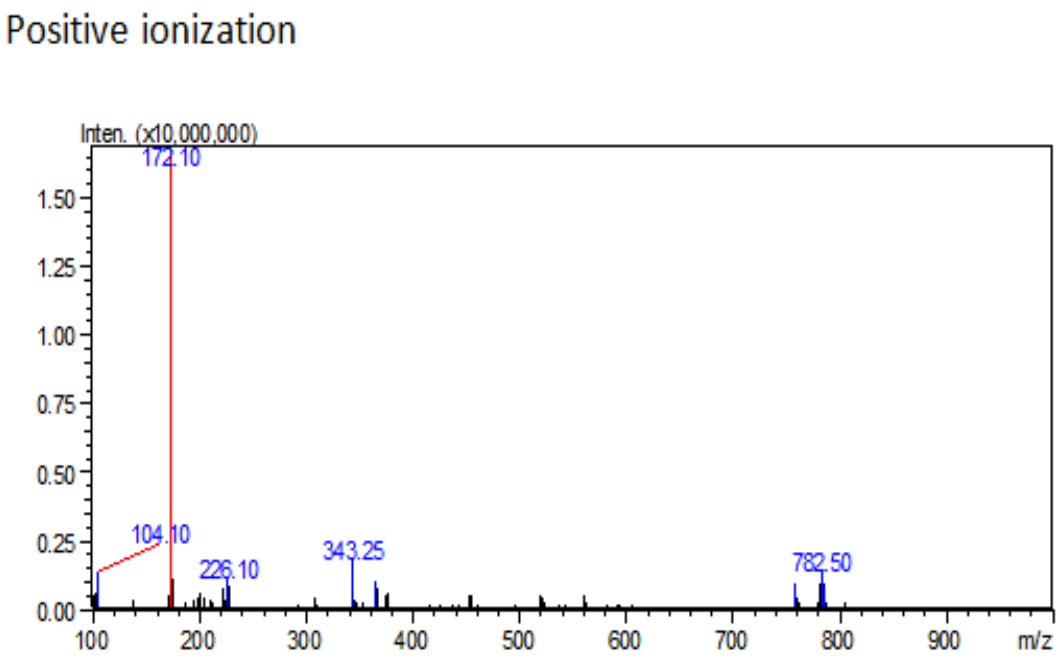


Figure 5. Mass spectra of Conjugate

The peak at 172 represents the presents of gabapentin, and the peak at 782 confirms the presence of the expected conjugate.

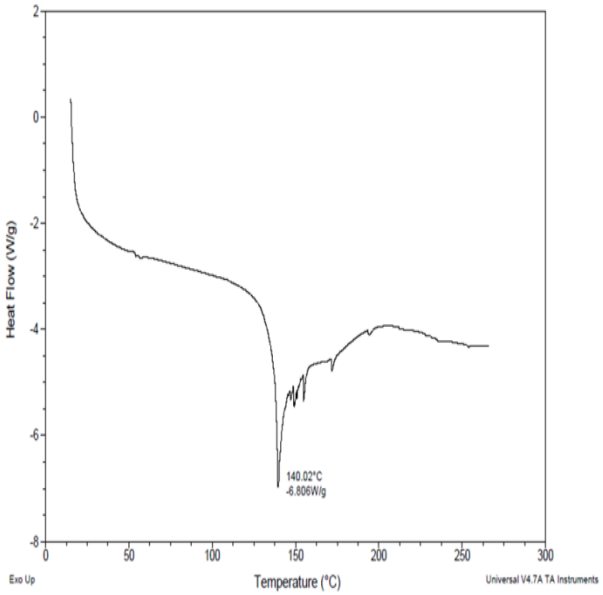
3.3 Pre-formulation studies

3.3.1 Solubility studies

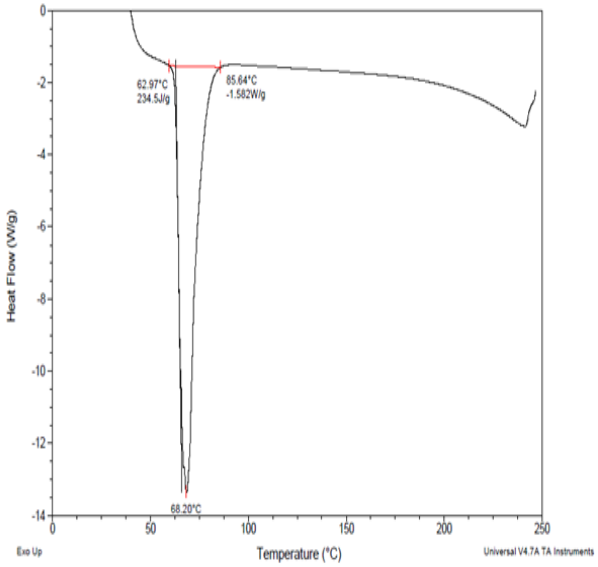
Oleic acid, Castrol oil, and linseed oil were absorbed in 0.97nm, 0.5nm, and 0.25nm, respectively. The concentration was determined, showing that the gabapentin-phosphatidylcholine conjugate is more soluble in oleic acid when compared with castor oil and linseed oil.

3.3.2 Compatibility studies: Differential scanning calorimetric studies

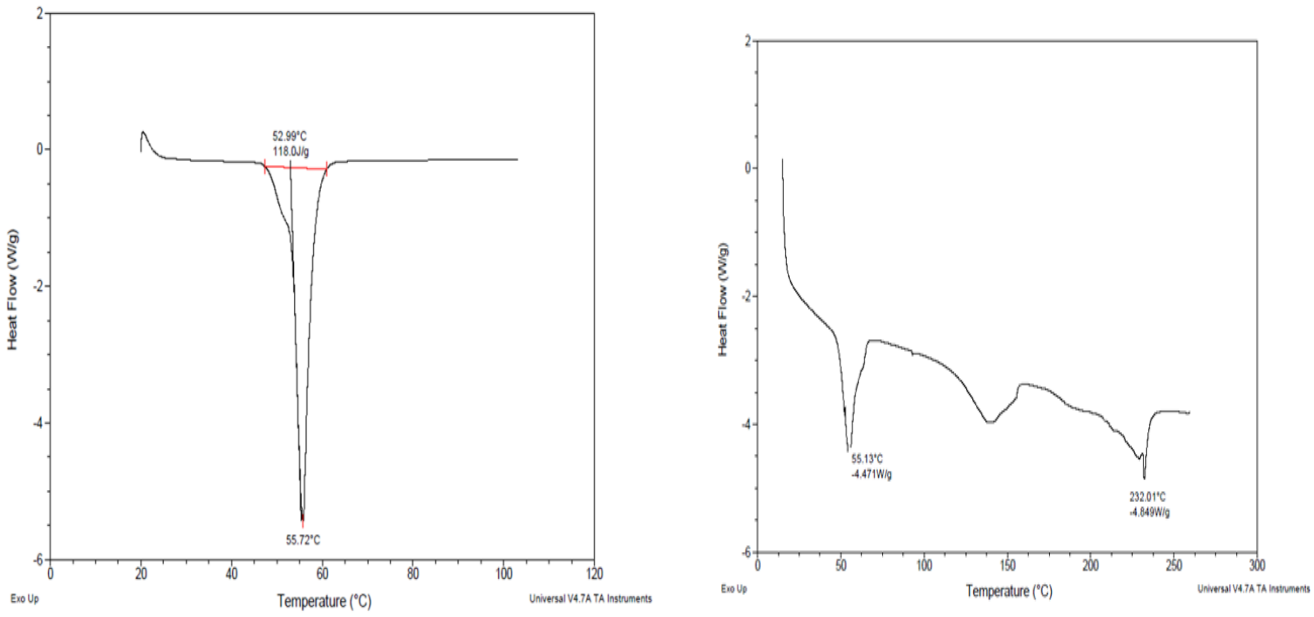
The appearance of the melting point peaks at the theoretical melting points of the compounds indicates the purity of the compound. The DSC curve of gabapentin appeared at 173^{0}C; phosphatidylcholine appeared at 123^{0}C; gabapentin- phosphatidylcholine conjugate appeared at 141^{0}C; palmitic acid appeared at 68^{0}C; pluronic F68 appeared at 55^{0}C. The peak for the physical mixture appeared respective to its melting point. It means that there is no interaction between the compounds.



6 a)



6 b)



6 c) 6 d)

Figure 6. a) DSC thermogram of the gabapentin-phosphatidylcholine conjugate, b) palmitic acid, c) pluronic F68, and d) physical mixture.

3.4 Formulation of NLC

The nanostructured lipid carriers were prepared using the m method. F4 and F6 were eliminated from the six batches prepared due to physical instability problems. The rest of the batches, F1, F2, F3, and F5, underwent characteristics analysis of nano-particle (particle size, zeta potential, and PDI analysis).

3.5 Evaluation of NLC

3.5.1 Particle size, Polydispersity index, and zeta potential

F1 and F2 show acceptable particle size and polydispersity index among the four batches. The particle size batches F3 and F5 are in the submicron level with a lesser polydispersity index when compared with F1 and F2. Some literature states that particle size less than 40 nm will have vascularity; it readily crosses any vesicle. A decrease in molecule size might be credited to a higher proportion of fluid lipids when contrasted with different groups. F1 is eliminated because of its particle size greater than 150nm. If the particle size is more than 150 Da, there will be an obstacle to targeting the brain.

The zeta potential exhibits the dimension of offensiveness between abutting, equivalently charged particles in dispersing. A high zeta potential will show steadfastness for iotas and particles that are sufficiently minimal; for instance, the course of action or dispersing will restrict collection. Interest outperforms repulsiveness when the potential is low, and the dispersing will break and flocculate. The stable formulations will have zeta potential ranges from -30 to +30. The zeta potential estimation of F1 and F2

are moderately close, - 4.21 and - 1.48, separately. F3 was disposed of because of its positive zeta potential esteem moreover.

A literature survey says that the positively charged nanoparticles show cytotoxicity for other factors in some cases. The negatively charged nanoparticles are eliminated more slowly from the blood than positively charged [20, 21]. It shows that selecting nano-formulation with negative zeta potential having more advantageous than positively charged particles.

Table 3. Particle size, PDI, and zeta potential report of various batches.

Batches	Particle size (nm)	PDI	Zeta potential (mV)
F1	178.5	0.428	-4.21
F2	50.08	0.472	-1.48
F3	42.06	0.247	0.910
F4	21.13	0.234	-0.325

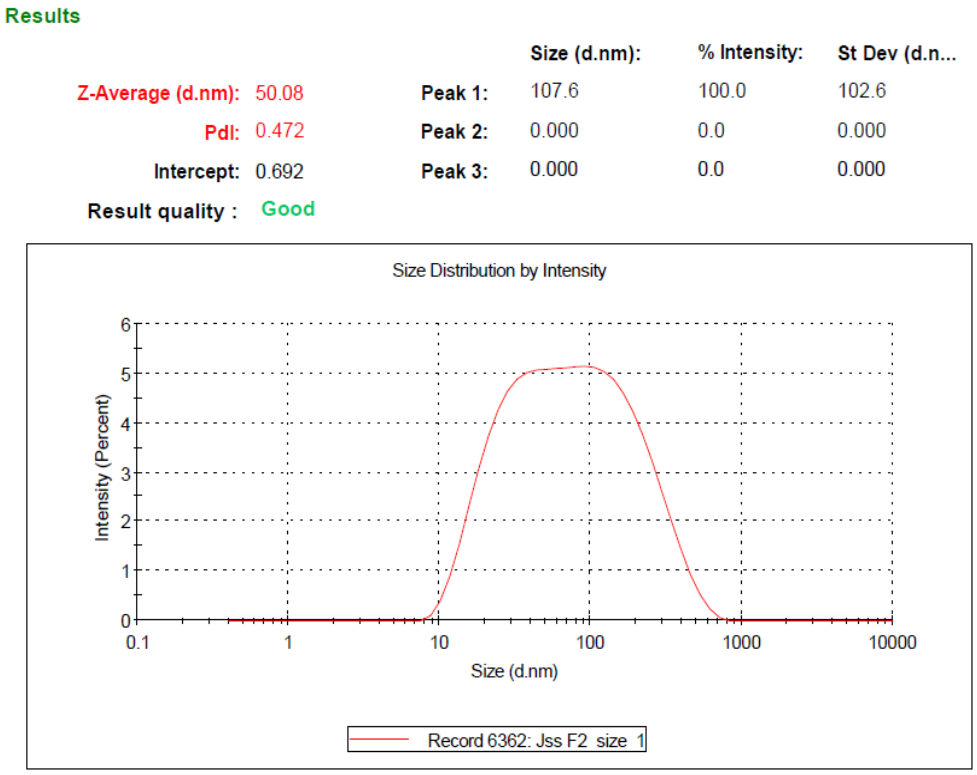


Figure 7: Particle size distribution & PDI report of F2

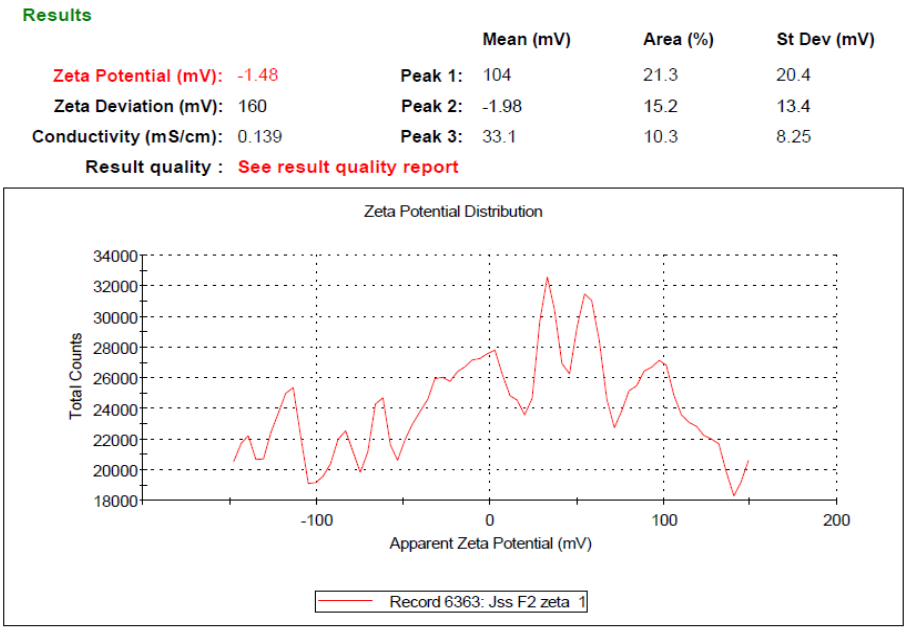


Figure 8: Zeta potential report of F2

2.5.2 Entrapment Efficiency

The entrapment of Gabapentin-phosphatidylcholine conjugate onto the NLC was controlled by investigating the supernatant with the expectation of complimentary medication utilizing a UV-Visible spectrophotometer at 220 nm after centrifugation of the drug-stacked NLCs. The information demonstrates the ensnarement productivity for all bunches. The entrapment efficiency of F1 and F2 was 81.26% and 77.8%, respectively.

2.5.3 In-vitro drug release and release kinetics

In vitro dissolution was done in phosphate buffer pH 7.4. The release profiles demonstrate that NLCs demonstrated an impeded arrival of the drug from the lipid matrix. It was seen that at the 48th hour, 65.58% were discharged from NLC. The NLC indicated continued drug release.

To clarify the mode of drug release, the in-vitro data was changed and interpreted at a graphical interface created using various kinetic models. The in vitro dissolution data for the NLCs in phosphate buffer was fitted into various models such as zero order, first order, Higuchi's, and Korsmayer- Peppa's. The best linearity was gotten in Higuchi's plot for NLC formulation showing that the release from the lattice is a square base of time subordinate procedure and as the 'n' value lies $0.5 < n < 1$ for mass transfer (0.899) follows a non-fickian model (anomalous transport).

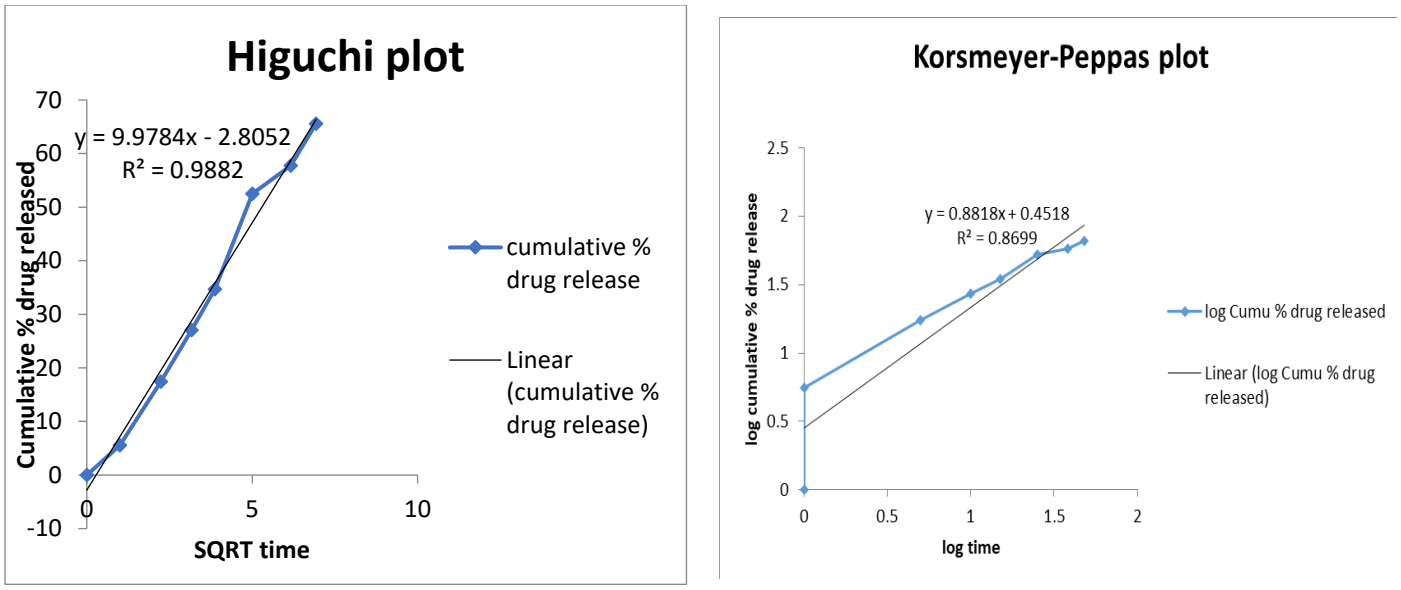


Figure 9: Higuchi's and Korsmeyer- Peppas plot for NLC

4. Conclusion

Since Gabapentin is a BCS Class III drug, having low permeability will turn to brain bioavailability issues. To overcome these issues, Gabapentin was conjugated with phosphatidylcholine. This conjugate may be targeted to phospholipase A2, and this enzyme will cleave the conjugate and release the gabapentin locally. This will lead to locally high concentrations of the drug in the brain. The conjugate was studied for the docking ability with phospholipase A2 using docking software Accelays Discovery studio 4.1 client, and it showed high binding affinity toward the enzyme with a docking score of -77.1084. Then the chemically conjugated gabapentin and phosphatidylcholine were loaded as NLCS, and its characterization was performed. The F2 batch has shown acceptable results in particle size, PDI, zeta potential, entrapment efficiency, and 65.58% drug release at the 48th hour. Release kinetics proves that the formulated NLCs follow Higuchi's release kinetics with an R^2 value of 0.9882, and the drug release from the NLCs was Non-Fickian diffusion (n value 899). This study concludes that Gabapentin phosphatidylcholine-loaded NLCs may be used to treat epilepsy.

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