DEVELOPMENT AND EVALUATION OF VALPROIC ACID LOADED NANOSTRUCTED LIPID CARRIER FOR ENHANCE BRAIN TARGETING

A. KRISHNANAND[1], ABHISHEK DEY[1], S. KAMESH[1], S. VIGNESH[1], N. JAWAHAR[1] AND V. SENTHIL[1]*

[1]Department of Pharmaceutics, JSS College of Pharmacy, JSS Academy of Higher Education & Research, Ooty - 643001, Tamil Nadu, India.

*Address for correspondence

Corresponding author – Dr. V. Senthil, M.Pharm., Ph.D.

Professor,

Department of Pharmaceutics

JSS College of Pharmacy, Ooty,

The Nilgiris - 643001

ABSTRACT:

Epilepsy is a neurological disorder with recurring as well as seizures. Nasal route: Since the BBB is impermeable to most of the drugs, delivering the drugs in therapeutically sufficient concentration into the brain is a tedious task. Many methods are being employed for brain targeting of the drugs. One of the most convenient routes among them is the nasal route of drug administration. This method proves efficient in delivering the drugs without causing damage to the BBB. There is a unique connection between the brain and the nasal route through which the drug can be delivered bypassing the BBB. Worldwide, there is a great influence of nanotechnology in pharmaceutical research groups from the initiation of the 20th century. Nanostructured Lipid Carrier (NLC) is the second generation of lipid nanoparticles having a structure like nanoemulsion. The unique advantages of NLC such as enhanced drug loading capacity and prevention of drug expulsion during storage make NLC is more favorable than Solid Lipid Nanoparticles (SLN). In addition to this, the usage of lipid carriers will increase the bioavailability and therapeutic effectiveness with low toxicity which can be acquired by changing the pharmacokinetics and biodistribution of the drugs. The prepared NLCs of

Valproic acid had a drug release of 66.12% in 72 hours and permeations of 26.55% in 6 hours. The permeation study results indicate that the formulation is capable of getting absorbed through the nasal route.

Keywords: Valproic acid, nanotechnology, NLC, nasal spray.

1. INTRODUCTION:

Epilepsy is a neurological disorder with recurring as well as seizures. Patients with one or two unprovoked seizures when they were diagnosed were not due to the identified and reversible clinical condition such as alcohol withdrawal or lowest blood sugar level.

In 1882, Burton synthesized the valproic acid (2-propylvaleric acid, 2-propylpentanoic acid, or n-dipropyl acetic acid) from valeric acid (a natural product of Valerian Officinalis) [1]. It is a short-chain and branched fatty acid, at room temperature it forms a clear liquid with a half-life of 9 – 16 hours. For organic compounds, it has been used as a "physiologically inert" solvent for almost a century. [2-4].

Valproate was scheduled under the succinic semi-aldehyde dehydrogenase inhibitor [5]. It causes a decrease in GABA metabolism and increases in GABAergic neurotransmission due to the increase in succinic semialdehyde dehydrogenase. An increase in GABA level causes increased inhibitory activity [6]. It contributes secondarily to cortical inhibition which leads to the inhibition of voltage-gated sodium channel activity and indirectly inhibition by the GABA effect.

Nasal route [7.8]. One of the most convenient routes among them is the nasal route of drug administration. This method proves efficient in delivering the drugs without causing damage to the BBB. There is a unique connection between the brain and the nasal route through which the drug can be delivered bypassing the BBB.

Drug administered through the nasal route reaches the brain mainly through two pathways [9.10]. They are the olfactory pathway and trigeminal nerves. These pathways provide a safe and effective method for brain drug delivery.

Worldwide, there is a great influence of nanotechnology in pharmaceutical technology research groups from the initiation of the 20th century even in all technical fields. There are different types of novel and advanced drug delivery technologies for lipophilic drugs for solving the solubility and stability issues of lipophilic formulations because the industries reported that around 40% of lipophilic formulations face solubility and stability issues [11].

NLCs are colloidal carriers in submicron size in the range of 40-1000 nm. The core of NLC consists of a combination of solid and liquid lipids. The unique advantages of NLC such as enhanced drug loading capacity and prevention of drug expulsion during storage make NLC is more favorable than Solid Lipid Nanoparticles (SLN) [12]. Recrystallization in NLC was less than SLN due to the crystalline order in NLC being disturbed by oil particles to maintain the system in the form of a liquid phase [13]. There were several methods to construct NLC such as High-Pressure Homogenization [14], O/W Microemulsion [15], Emulsification-solvent evaporation, multiple emulsion water/oil/water [16]. The high shear homogenization and/or ultrasonication were a dispersion technique that did not use an organic solvent, a large amount of surfactant, or an additive compound. The melting lipid was added and dispersed in a solution of surfactant using the ultrasonication method [17]. by probe sonicator [18].

The drug delivery across various routes such as oral, parenteral, topical, pulmonary, and ophthalmic routes due to their physicochemical properties like size, shape, biocompatibility, safety, capacity to compress a variety of drugs, and pliability for controlling the drug release over the advantage of regulatory compliance of excipients used, scale-up practicability and commercial possibility. In addition to this, the usage of lipid carriers will increase the bioavailability and therapeutic effectiveness with low toxicity which can be acquired by changing the pharmacokinetics and biodistribution of the drugs [19].

2.1 MATERIALS:

Table 1: list of materials

S. No	Material
1.	Valproic acid
2.	Stearic acid
3.	Oleic acid
4.	Tween 80
5.	Poloxamer 188
6.	Soy lecithin
7.	Acetonitrile
8.	Sodium Hydroxide
9.	Potassium dihydrogen phosphate

2.2 PRE-FORMULATION STUDIES

Pre-formulation studies involve the collection and understanding of the chemical and physical properties of the chemical entities which are used in the formulation. Pre-formulation studies include the data collection of both the APIs and the excipients. Pre-formulation studies in the formulation of a dosage form involve selection of excipients required for the formulation, screening of the excipients, tabulation of the physicochemical properties, finding the compatibilities and incompatibilities of the excipients with the drug. Pre-formulation is an important step in the development of safe, stable, and efficacious drug formulations. Preformulation studies involved in this study are lambda max determination, preparation of calibration curve, solubility studies, compatibility studies involving FT-IR and DSC.

2.2.1 Determination of λ max Valproic acid

Lambda max (λmax) determination of the drug was determined using UV spectroscopy. UV 1700, Shimadzu, (Kyoto, Japan) was used for the determination of the λmax. Accurately measured 1.13ml of the drug valproic acid was dissolved in 100ml of water containing 0.5ml of tween 80. The resultant solution contains 1mg per ml solution of valproic acid. The solution if further diluted to obtain 100µg/ml solution of valproic acid. The spectrum was obtained by scanning the solution from 400nm to 200nm[20].

2.2.2 Solubility

The solubility of valproic acid was tested in different solvents alone and a mixture of solvents. A measured volume of about 0.1ml of the drug valproic acid was dissolved in 10ml of water, water-tween 80(95:5) mixture, ethanol, methanol [21], water: acetonitrile (55:45) mixture, PBS buffer. Solubility was determined based on physical appearance.

2.2.3 Development of Calibration curve

An accurate volume of 1.13ml of the drug valproic acid was dissolved in 100ml water-tween 80 mixture. 1.13ml of valproic acid was transferred to a 100ml volumetric flask. To the volumetric flask 0.5ml tween 80 was added, and different the volume was made up to 100ml using water. The made-up solutions were diluted further and, concentrations of 2µg, 4µg, 6µg, 8µg, and 10µg were prepared. The samples were checked in by UV apparatus to determine the

absorbance. Samples were estimated at the λmax obtained against the blank. The standard curve was plotted with concentration against the absorbance.

2.2.4 Compatibility studies

Compatibility studies were conducted using FTIR and DSC methods.

2.2.4.1 Fourier Transform Infra-Red Spectroscopy FTIR [23]

FTIR was used for the analysis of the compatibility of the drug with the excipients. Samples of the drug, liquid lipid, solid lipid, and the physical mixture were analyzed for determining the compatibility. The solid samples were ground in mortar and pestle along with KBr in the ratio of 1:4. KBr and solid samples were pressed into solid pellets using a hydraulic press at 10,000psi pressure. And the liquid samples were dissolved in chloroform and were placed on KBr Pellets. The samples were analyzed from wave number 400cm^{-1} to 4000cm^{-1}. The obtained spectra were compared for evaluating the disappearance and shifting of functional groups.

2.3 Preparation of Valproic acid loaded NLCs

Valproic acid NLCs are prepared using the hot homogenization method. The method uses 2% of the total formulation as lipids. An organic phase containing stearic acid as solid lipid, oleic acid as liquid lipid, valproic acid drug, and soy lecithin were mixed in 100ml of 1:1 ratio of methanol, and chloroform was added to a round bottom flask. And the round bottom flask was connected to a rotary vacuum evaporator at 63°C temperature and 100rpm speed. The round bottom flask was rotated for about 40mins till the solvents completely evaporates and forms a film on the surface of the round bottom flask. The aqueous phase containing poloxamer and tween 80 was mixed in 200ml water, it was heated to 70°C temperature. The temperature was checked using a thermometer. The aqueous phase was added to the organic phase. The resultant solution was mixed at 35,000rpm using a high-speed homogenizer for 5 minutes. The nanoemulsion such formed is kept in a sonicator for 20 minutes. The formulation was kept overnight for stress releasing of the formed NLCs. They are then centrifuged at 10,000 rpm for 3 hours.

2.3.1 Freeze Drying

The prepared NLCs after centrifuging were mixed with 10% mannitol solution and it was poured into a freeze dryer pan. These pans were covered with aluminum foils and kept inside

the deep freezer for 24 hours. After 24 hours of freezing samples were kept lyophilized at -89°C for 24hours, and finally Dried powder was obtained.

2.3.2 Optimization

Optimization was done by 3 level 2 factorial method. The concentrations of poloxamer 188, soy lecithin, and drug were optimized based on the literature review. The processing factors were also confirmed based on the literature review. Here, concentrations of stearic acid, oleic acid, and tween 80 were selected as dependant variables, and particle size, zeta potential, polydispersity index, and entrapment efficiency were determined as independent variables. These data were optimized using Design-Expert® 12 by Stat-Ease. The software provided 9 randomized formulas for the preparation of trial batches.

Table 2: Summary of the randomization

File Version	12.0.7.0	-	-	--
Study Type	Response Surface	-	**Subtype**	Randomized
Design Type	D-optimal	Best	**Runs**	9.00
Design Model	Reduced Quadratic	-	**Blocks**	No Blocks

Table 3: Factors and levels assigned for them.

Factors:

Factor	Name	Units	Type	Min.	Max.	Coded Low	Coded High	Mean	Std.Dev ±
A	LL:SL		Numeric	20.00	50.00	-1↔20.00	-1↔50.00	35.00	12.99
B	Tween 80	%	Numeric	10000	2.00	-1↔1.00	-1↔2.00	1.50	0.4330

Table 4: Randomised formulas as per design expert.

Std.	Run	Block	Stearic acid	Oleic acid	Tween 80
7	1	Block 1	80	20	2
4	2	Block 1	80	20	1.5
2	3	Block 1	65	35	1
9	4	Block 1	50	50	1.5
6	5	Block 1	50	50	1
3	6	Block 1	50	50	1.5
1	7	Block 1	80	20	1
5	8	Block 1	65	35	2
8	9	Block 1	65	35	1

2.3.2.1 3D response surface plots

The 3D response surface plots of the optimization depicting the interaction are prepared using the Design-Expert® 12 by Stat-Ease.

2.3.2.2 Desirability criteria

Desirability is the depiction of the closeness of the observed result to the ideal value. Desirability was used for optimizing the independent variables. Desirability should lie between 0 and 1. The desirability is the "geometric mean of the independent variables".

2.4 Characterisation of Valproic acid-loaded NLCs [23].

2.4.1 Particle size and Polydispersity index

Particle size and particle size distribution were determined using Anton Paar litesizer 500. Glass cuvettes were used in Anton Paar litesizer 500 for the estimation of particle size and Polydispersity index.

2.4.2 Zeta potential

Zeta potential was also determined using Anton Paar litesizer 500. Omega Cuvettes were used in Anton Paar litesizer 500 and evaluations were done at 75mV.

2.4.3 Entrapment efficiency

The prepared NLCs were centrifuged at 10,000rpm for 3 hours. The Supernatant obtained is decanted. From this, an aliquot is taken and diluted approximately and the concentration is estimated.

The total drug content in the supernatant was calculated. This quantity is subtracted from the total quantity of drug added during the formulation. This gives the amount of drug entrapped.

% EE =amount of drug added− the amount of drug in supernatant x100 ---------- Equation 1

2.4.4 Light microscopy

A small quantity of the formulation is kept on a glass slide and is observed under a light microscope for confirming the formation of NLCs. The focus and light are adjusted such that a clear image is obtained.

2.5 Evaluation of valproic acid loaded NLCs

2.5.1 *In-vitro* drug release study [24].

The dialysis membrane method was applied for *in-vitro* drug release of the valproic acid-loaded NLCs. The prepared formulation was diluted to 5ml using simulated nasal fluid (SNF). Simulated nasal fluid was prepared by dissolving sodium chloride, potassium chloride, and calcium chloride in water. The cellophane membrane or the dialysis membrane was tied on one side of a cylindrical tube. And the tube was kept in contact with the solvent in the acceptor compartment. The acceptor compartment was filled with 200ml of SNF. The drug formulation equivalent to 50mg drug, diluted to 5ml was added to the tube which acts as a donor compartment. The whole system was maintained at 37±0.5°C using a temperature-controlled magnetic stirrer. Magnetic bead stirring at a rate of 100rpm was used inside the acceptor compartment to provide constant stirring. An aliquot of 1ml of the solution was withdrawn from the acceptor compartment at 5mins, 10mins, 15mins, 30mins, 1 hour, 2 hours, 4hour, 6hour, 8hour, 12hour, 24hour, 48hour and 72-hour intervals. The samples were made up to 10ml in a 10ml volumetric flask using simulated nasal fluid. The filter sample was evaluated in UV spectroscopic equipment.

2.5.2 *In-vitro* release kinetics: *In-vitro* release kinetics are important in the development of drug formulation. The release mechanism was found out by applying the data obtained to various kinetic models.

2.5.2.1 Zero-order

Zero-order release gives the overall dissolution pattern of the active pharmaceutical ingredient from the different types of modified drug release dosage forms.

Equation:

$Qt = Q_0 + K_0t$ ---------- Equation 2

Where Qt = Quantity of release of drug in time 't'

Q_0 = Initial quantity of drug in solution

2.5.2.2 First Order

Firs order release gives details of the active pharmaceutical ingredients within the system. This is concentration dependant release. Equation 3:

Log Qt = logQ0 + K1t/2.303---------- Equation 3

Where Qt = Amount of drug release in time t

Q = Initial amount of drug in solution

K = Constant

2.5.2.3 Higuchi's Model

Higuchi's model demonstrates drug release from the matrix which is insoluble as a square root of time. Equation 4:

$Qt = KH \sqrt{t}n$ ---------- Equation 4

Q = Cumulative amount of drug release at time't'

KH = Higuchi Constant

2.5.2.4 Korsmeyer's- Peppa's Model

This model gives a simple derivation. It describes a drug release from the polymeric system. Equation 5:

$Mt/M\infty = K_{kp}t^n$ ---------- Equation 5

Where $Mt/M\infty$ is a fraction of drug released at time t,

$\log(Mt/M\infty)=\log K_{kp}+n\log t$,

Mt is the amount of drug released in time t,

M∞ is the amount of drug released after time ∞,

n is the diffusional exponent or drug release exponent,

Kkp is the Korsmeyer release rate constant.

2.5.3 *Ex-vivo* Permeation studies

Ex-vivo permeation studies were conducted on isolated goat mucosa. A thin layer of mucosa tissues is obtained. Franz-diffusion apparatus is employed for estimation of *ex-vivo* permeation of the drug. Formulation equivalent to 50mg of valproic acid is diluted to 3ml with simulated nasal fluid. The donor compartment is filled with the drug solution and the acceptor compartment is filled with SNF. Goat mucosa is kept in between the compartment and is assembled such that there is no space in between to avoid leakage. Temperature is maintained at 37±5°C using an external bath. An aliquot of 1ml is withdrawn at 10min. 30 min, 1hr, 2,3,4,5 and 6hrs. The obtained sample is diluted appropriately and estimated using UV Spectrophotometer.

3. RESULTS AND DISCUSSION:

3.1 Preformulation studies

3.1.1 Determination of λ_{max} of Valproic acid

Valproic acid was dissolved in a water-tween 80(1ml in 100ml) mixture and was diluted to approximately a concentration of 100µg/ml. The solution was scanned from 400nm to 200nm in UV 1700, Shimadzu, and (Kyoto, Japan).

Maximum absorbance was obtained at 214.5nm which comes very close to the value suggested by USP, So, we confirmed the obtained substance is valproic acid.

6.1.2 Solubility

Solubility of valproic acid was tested in water, water-tween 80 mixture, ethanol, methanol, water: acetonitrile mixture, PBS buffer. Water-tween 80 mixture is optimum for the preparation of the formulation since the ingredients used contain both water and tween 80.

Table 5: Solubility of the drug in different solvent systems

Solvent	Solubility
Water	Insoluble
Water-tween 80 mixture	Freely soluble
Ethanol	Freely soluble
Methanol	Freely soluble
Water: acetonitrile mixture	Freely soluble
PBS buffer	Insoluble
PBS buffer-tween 80 mixture	Freely soluble

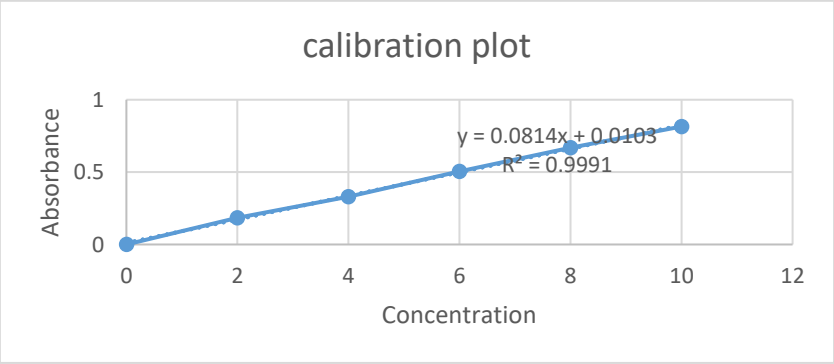
3.1.3 Development of Calibration Curve

Table:9 shows absorbance data observed for different concentrations used for the development of calibration curve of Valproic acid using PBS buffer-tween 80 mixture.

Table 6: Concentration versus absorbance data observed for development of calibration curve

Concentration µg/ml	Absorbance
0	0
2	0.185
4	0.331
6	0.505
8	0.668
10	0.815

Figure 1: Calibration curve of valproic acid



The calibration curve of valproic acid was developed from the absorbance of the different concentrations and a perfect correlation was obtained after plotting the values in the graph. The regression value (R^2=0.9991) depicts that there is perfect linearity for the valproic acid in the range of 2µg/ml to 10 µg/ml. The observed data are shown in **Table 6.** The linearity curve is shown in **figure 1.**

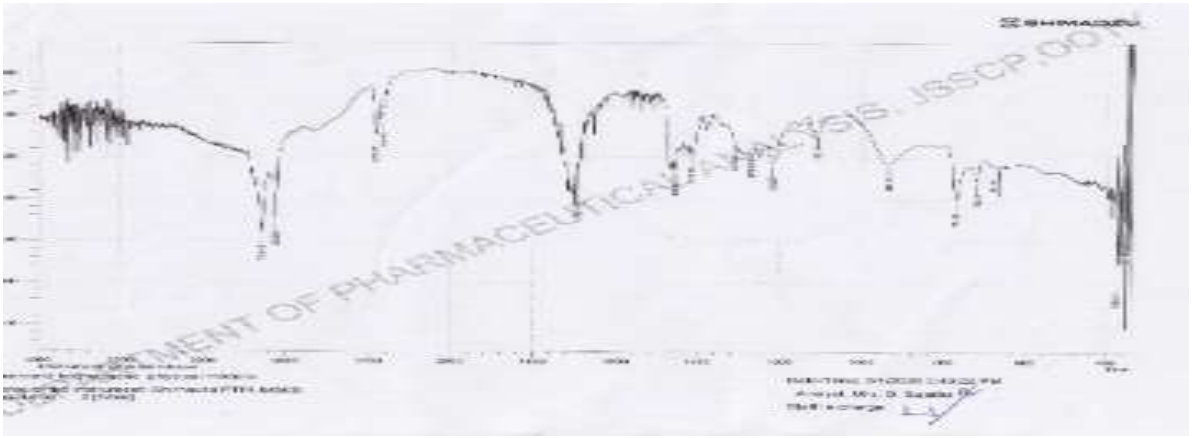
3.1.4 Compatibility Studies

3.1.4.1 Fourier Transform Infra-Red

The compatibility of the valproic acid with the excipients was tested. The spectra obtained were analyzed to find out whether there is any loss or shifting of the functional group in the physical mixture. It was observed that there is no loss of any functional group or there is no major shifting in peaks. It establishes that the valproic acid is compatible with excipients used.

From the FTIR data, we can observe that there is no major shift or any deletion in the functional group of the drug which means there is no major interaction between the drug and the excipients.

Figure 2: FTIR result of physical mixture of 1:1:1 mixture of valproic acid, stearic acid, and oleic acid



3.2 Optimization Factor 1 was selected as the ratio between solid lipid and liquid lipid. Factor 2 was selected as the concentration of tween 80. Each factor is assigned 3 levels with high low and medium levels. The responses were selected as particle size, entrapment efficiency, and zeta potential Polydispersity index. The trials were performed based on the formulas suggested by the software. The values were entered into the software. Fit summary, model summary ANOVA were performed. Then the model graphs were generated. Appropriate models were

chosen after these tests. Adequacy of the model was selected to maximize the zeta potential, entrapment efficiency and to minimize the PDI and particle size.

Table 7: The layout shows the values of dependant variables and their responses for the 9 formulations

Run	Factor 1		Factor 2				
	Stearic acid	Oleic acid	Tween 80	Particle size nm	Entrapment efficiency %	Zeta potential mV	PDI
1	80	20	2	513.8nm	90.65833	-16.5	32.80%
2	80	20	1.5	234.1nm	93.2423	-15.9	26.50%
3	65	35	1	119.4nm	91.7219	-19.2	29.70%
4	50	50	1.5	120.82nm	90.326	-17.7	22.80%
5	50	50	1	176.47nm	90.9848	-18.4	17.70%
6	50	50	1.5	193.23nm	89.92	-17.6	26.90%
7	80	20	1	197.29nm	93.1406	-17.2	26.90%
8	65	35	2	457.0nm	95.4287	-16.5	44.40%
9	65	35	1	168.02nm	93.2045	-17.9	24.00%

3.2.1 Fit summary for each response

Table 8: Fit summary of response 1: particle size

Source	Model p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	-
Design model	0.258	-	0.8082	0.3202	Recommended
Linear	**0.0163**	**-**	**0.6619**	**0.3730**	**Suggested**
2FI	0.1557	-	0.7395	0.4272	-
Quadratic	0.4455	-	0.7468	-0.0959	-
cubic	0.3696	-	0.8963	-1.3634	Aliased

Table 9: Fit summary of response 2: Polydispersity Index

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	-
linear	0.6683	-	-0.1657	-0.6631	-
2FI	0.9624	-	-0.3982	-2.0110	-
Quadratic	**0.0180**	**-**	**0.8401**	**0.4681**	**Suggested**
cubic	0.8359	-	0.6648	-6.6368	Aliased

Table 10: Fit summary of response 3: Zeta potential

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	-
linear	0.3259	-	0.0825	-0.3004	-
2FI	1.0000	-	-	-	-
Quadratic	**0.0102**	**-**	**0.9135**	**0.7455**	**Suggested**
cubic	0.9563	-	0.7626	-4.4076	Aliased

Table 11: Fit summary of response 4: Entrapment efficiency

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	-
Mean	**<0.0001**	**-**	**-**	**-**	**Suggested**
Linear	0.4395	-	-0.0138	-0.8568	-
2FI	0.5507	-	-0.1246	-2.6563	-
Quadratic	0.4055	-	-0.0269	-3.5488	-
Cubic	0.2824	-	0.7544	-4.5960	Aliased

3.2.2 ANOVA analysis for each model selected for each response

Table 12: ANOVA for Reduced Quadratic model

Response 1: Particle Size

Source	Sum of Squares	df	Mean Square	F-value	p-value	-
Model	1.483E+05	4	37073.00	9.43	0.0258	significant
A-LL:SL	34454.13	1	34454.13	8.76	0.0416	-
B-Tween 80	87971.89	1	87971.89	22.37	0.0091	-
AB	14896.20	1	14896.20	3.79	0.1235	-
B^2	10969.77	1	10969.77	2.79	0.1702	-
Residual	15730.23	4	3932.56	-	-	-
Cor Total	1.640E+05	8	-	-	-	-

Factor coding is **coded**.

The Sum of squares is **Type III - Partial**

The **Model F-value** of 9.43 implies the model is significant. There is only a 2.58% chance that an F-value this large could occur due to noise. P-**values** less than 0.0500 indicate model terms are signifiant.

Table 13: ANOVA for Quadratic model

Response 2: PDI

Source	Sum of Squares	df	Mean Square	F-value	p-value	-
Model	49.36	5	9.87	9.41	0.0472	Significant
A-LL:SL	6.00	1	6.00	5.72	0.0967	-
B-Tween 80	0.6017	1	0.6017	0.5733	0.5040	-
AB	0.0225	1	0.0225	0.0214	0.8929	-
A^2	41.71	1	41.71	39.74	0.0081	-
B^2	1.03	1	1.03	0.9787	0.3954	-
Residual	3.15	3	1.05	-	-	-
Cor Total	52.51	8	-	-	-	-

Factor coding is **coded**.

The Sum of squares is **Type III – Partial**

The **Model F-value** of 9.41 implies the model is significant. There is only a 4.72% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant.

Table 14: ANOVA for Quadratic model

Response 3: Zeta Potential

Source	Sum of Squares	df	Mean Square	F-value	p-value	-
Model	10.47	5	2.09	17.89	0.0193	significant
A-LL:SL	3.37	1	3.37	28.84	0.0126	-
B-Tween 80	0.0000	1	0.0000	0.0000	1.0000	-
AB	0.0000	1	0.0000	0.0000	1.0000	-
A^2	7.09	1	7.09	60.61	0.0044	-
B^2	0.0022	1	0.0022	0.0190	0.8991	-
Residual	0.3511	3	0.1170	-	-	-
Cor Total	10.82	8	-	-	-	-

Factor coding is **coded**.

The Sum of squares is **Type III - Partial**

The **Model F-value** of 17.89 implies the model is significant. There is only a 1.93% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant.

Table 15: ANOVA for Mean model

Response 4: EE

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	0.0000	0	-	-	-
Residual	27.10	8	3.39	-	-
Cor Total	27.10	8	--	-	-

Factor coding is **coded**.

The Sum of squares is **Type III - Partial**

P-values less than 0.0500 indicate model terms are significant.

6.2.3 Fit statistics tables for each factor

Table 16: Fit Statistics for particle size

Std. Dev.	62.71	R^2	0.9041
Mean	242.24	Adjusted R^2	0.8082
C.V. %	25.89	Predicted R^2	0.3202
-	-	Adeq Precision	8.7231

Table 17: Fit Statistics for polydispersity Index

Std. Dev.	1.02	R^2	0.9400
Mean	27.59	Adjusted R^2	0.8401
C.V. %	3.71	Predicted R^2	0.4681
-	-	Adeq Precision	7.8006

Table 18: Fit Statistics for zeta potential

Std. Dev.	0.3421	R^2	0.9676
Mean	-17.44	Adjusted R^2	0.9135
C.V. %	1.96	Predicted R^2	0.7455
-	-	Adeq Precision	9.5467

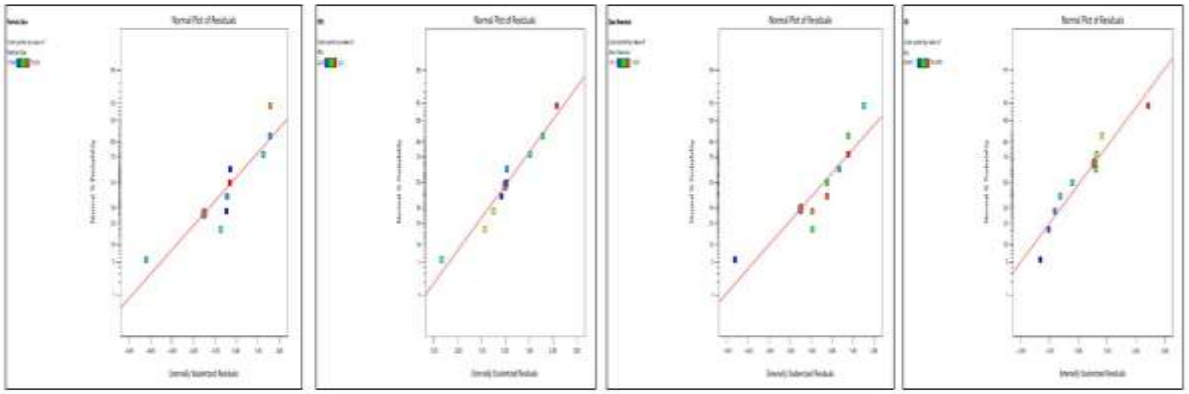
The **Predicted R^2** of 0.7455 is in reasonable agreement with the **Adjusted R^2** of 0.9135; i.e. the difference is less than 0.2.

Table 19: Fit Statistics entrapment efficiency

Std. Dev.	1.84	R^2	0.0000
Mean	92.12	Adjusted R^2	0.0000
C.V. %	2.00	Predicted R^2	-0.2656
-	-	Adeq Precision	NA(1)

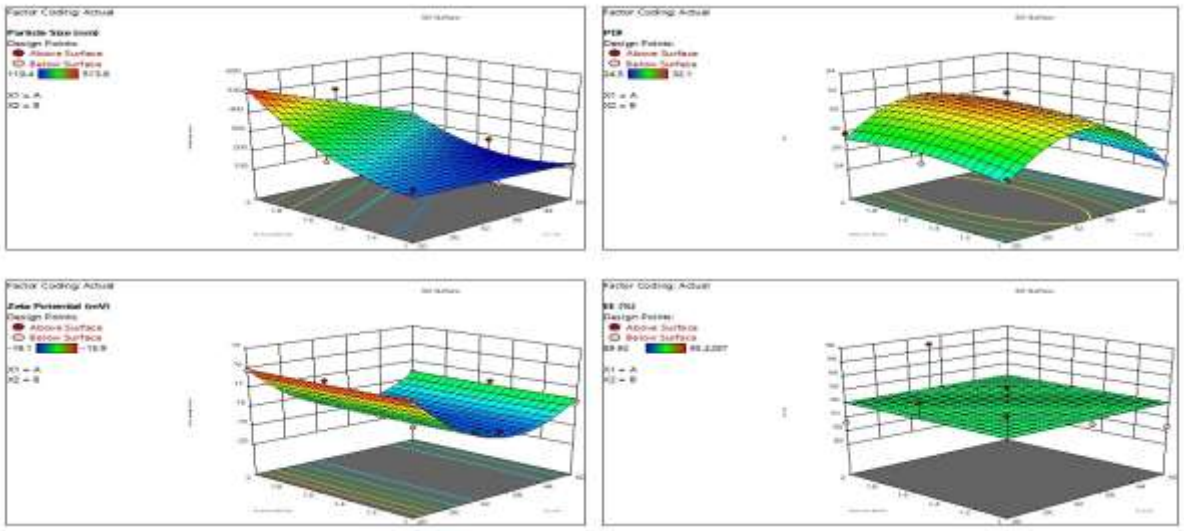
(1) Case(s) with leverage of 1.0000: Pred R^2 and PRESS statistic not defined.

Figure 3: From left to right the Normal plot of residuals for A, (particle size) B, (Polydispersity Index), C, (Zeta potential Figure), D (Entrapment efficiency).



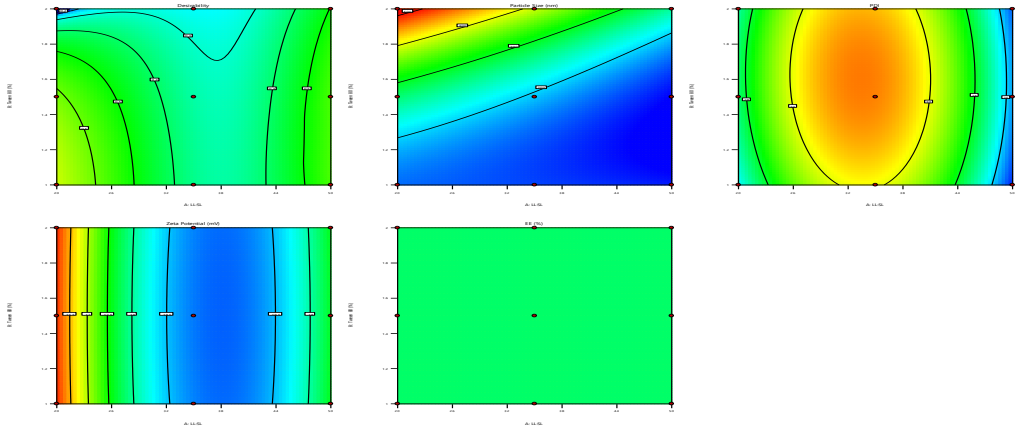
3.2.5.1 3D Response Surface plots

In a clockwise manner - Figure (A) is the 3D response surface plot showing the relation between the factors and response 1 (particle size), Figure (B) response 2 (Polydispersity index), Figure (C) response 4 (Entrapment efficiency), Figure (D) response 3 (Zeta potential) is shown in Figure 4.



3.2.5.2 Contour plot of the optimized results and desirability

Figure 5: Contour plot indicating the desirability and optimized values for all the responses.



3.2.5.3 Overlay plot

Figure 6: Overlay plot depicting the optimized formula with expected results

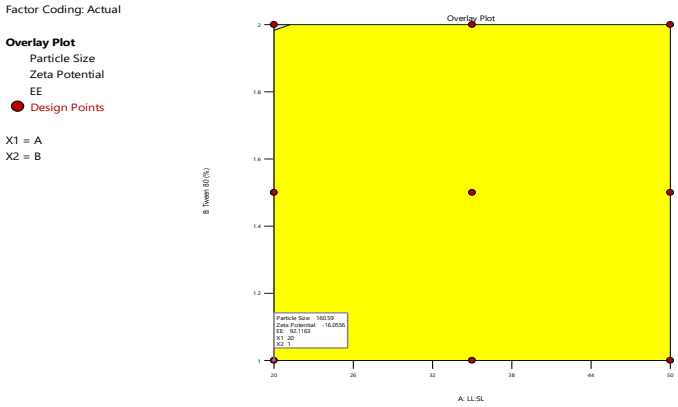


Table 20: Optimised formula for the Factors

Factor	Name	Low Level	High Level	Std. Dev.	Coding
A	**LL: SL**	**20.00**	**50.00**	**0.0000**	**Actual**
B	**Tween 80**	**1.0000**	**2.00**	**0.0000**	**Actual**

Table 21: Confirmation table of the predicted values

Two-sided Confidence = 95%

Solution 1 of 11	Predicted Mean	Predicted Median	Std. Dev.	n	SE Pred.	95% PI low	Data Mean	95% PI high

Response								
Particle Size	119.399	119.399	62.7101	1.0000	78.7917	-99.3619	142.5	338.16
PDI	24.8244	24.8244	1.02447	1.0000	1.30964	20.6565	22.6	28.9923
Zeta Potential	-17.5649	-17.5649	0.342107	1.0000	0.437336	18.9567	-16.8	-16.1731
EE	92.1163	92.1163	1.84047	1.0000	1.94003	87.6426	92.12	96.59

Table 22: Point Prediction based on optimisation

Two-sided Confidence = 95% Population = 99%

Response	Predicted Mean	Predicted Median	Std Dev	SE Mean	95% CI low for Mean	95% CI high for Mean	95% TI low for 99% Pop	95% TI high for 99% Pop
Particle Size	119.39	119.39	62.71	47.70	-13.04	251.84	-365.50	604.30
PDI	24.82	24.82	1.02	0.81	22.22	27.42	15.19	34.44
Zeta Potential	-17.56	-17.56	0.34	0.27	-18.43	-16.69	-20.77	-14.35
EE	92.11	92.11	1.84	0.61	90.70	93.53	82.86	101.36

Table 23: Coefficient table

		Intercept	A	B	AB	A^2	B^2
Particle Size		192.863	-75.7783	121.087	-61.025	-	74.06
p-values		-	0.0416	0.0091	0.1235	-	0.1702
PDI		31.1111	-1	0.316667	-0.075	-4.56667	-0.716667
p-values		-	0.0967	0.5040	0.8929	0.0081	0.3954
Zeta Potential		-18.7222	-0.75	-1.4189E-15	2.16379E-16	1.88333	0.0333333
p-values		-	0.0126	1.0000	1.0000	0.0044	0.8991
EE		92.1163	-	-	-	-	-
p-values		-	-	-	-	-	-

3.3 Optimized formula for final formulation

Table 24: Optimized formula for final formulation 200ml

S.No	Material	Concentration
1	Valproic acid	3ml
2	Stearic acid	50%
3	Oleic acid	50%
4	Tween 80	1%
5	Poloxamer	1%
6	Soy lecithin	1%
7	Methanol	50ml
8	Chloroform	50ml

3.4 Characterisation of Valproic acid

3.4.1 Particle size and PDI

Particle size is considered an important factor that determines the brain permeability of the drug. It is optimum to have a particle size less than 200nm to pass the BBB. As per the resulting

system of Anton Paar litesizer 500, the PDI values are represented in percentage. Less than 20% is considered optimum and 20-10% is considered acceptable.

The particle size and PDI of the formulation were estimated and the results were found to be 142.5nm and 22.6% respectively. This falls in the acceptable range. The particle size and PDI estimation report of the formulation are attached below.

3.4.2 Zeta potential

The zeta potential is an important factor that determines the stability of the nanoformulation. Higher the value more the stability for the nanoformulation. The value can be either negative or positive. The Zeta potential of the formulation was estimated to be -16.8mV. The zeta potential estimation report is attached below.

Figure 7: Particle size distribution chart for the formulation

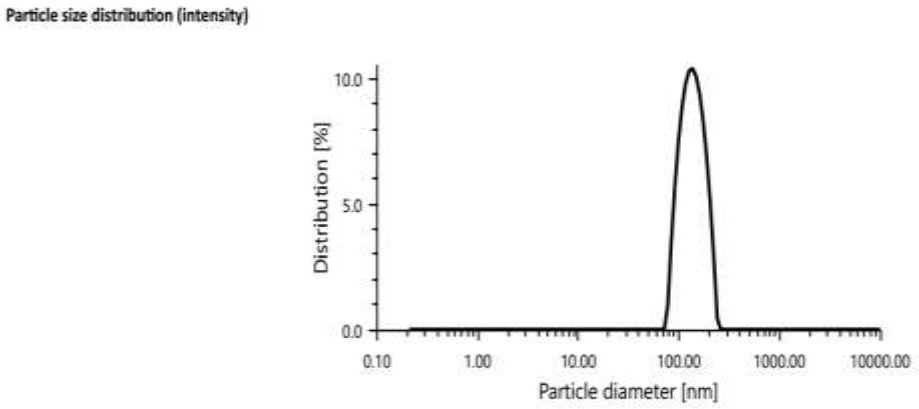
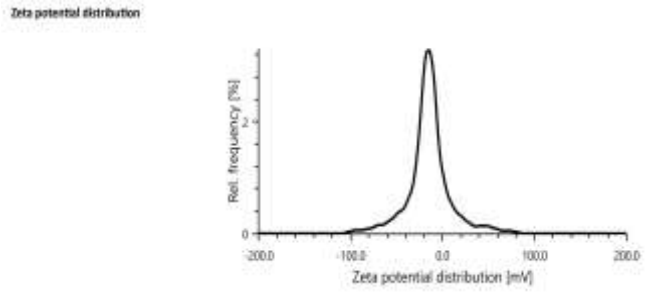


Figure 8: Zeta potential distribution chart for the formulation



3.4.3 Entrapment efficiency

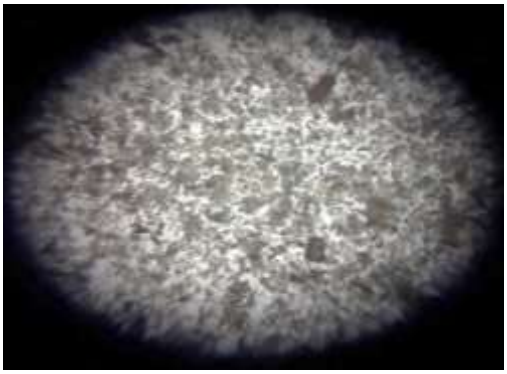
The entrapment efficiency of the formulation is important in calculating the equivalent quantity of formulation for further evaluations such as *in-vitro* studies and *ex-vivo* studies. Entrapment efficiency for the final formulation was found to be 92.12%w/v.

3.4.4 Morphological analysis

3.4.4.1 Light microscopy

The light microscopic image of the dilute solution of prepared suspension confirms the formation of NLCs.

Figure 9: Optical light microscopic image of the formulated NLCs



3.5. Evaluation of valproic acid loaded NLCs

3.5.1 *In-vitro* Drug release profile

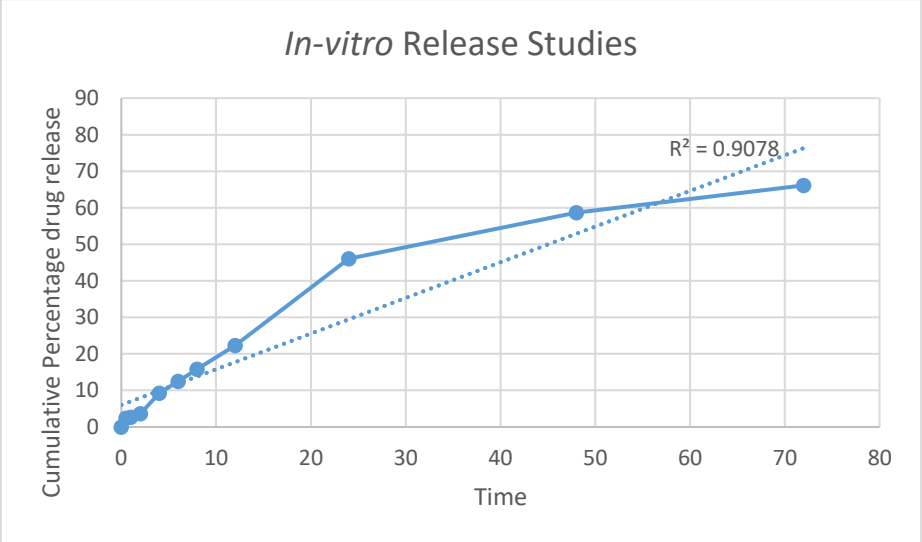
In-vitro drug release testing was performed using a simulated nasal fluid with pH 6.4. The drug release was calculated for 72hrs in the SNF. The cumulative drug release for 72hrs was found to be 66.12%. A sustained drug release mechanism was observed. A controlled drug release mechanism was observed.

Figure 10: modified *In-vitro* drug release study assembly

Table 25: Cumulative percentage drug release for the drug in Simulated Nasal fluid

Sl.No.	Time in Hours	Cumulative % Drug Release
1	0	0
2	0.5	2.4534
3	1	2.7001
4	2	3.6842
5	4	9.2419
6	6	12.4638
7	8	15.8213
8	12	22.2753
9	24	46.0913
10	48	58.6918
11	72	66.1252

Figure 11: Cumulative drug release graph.



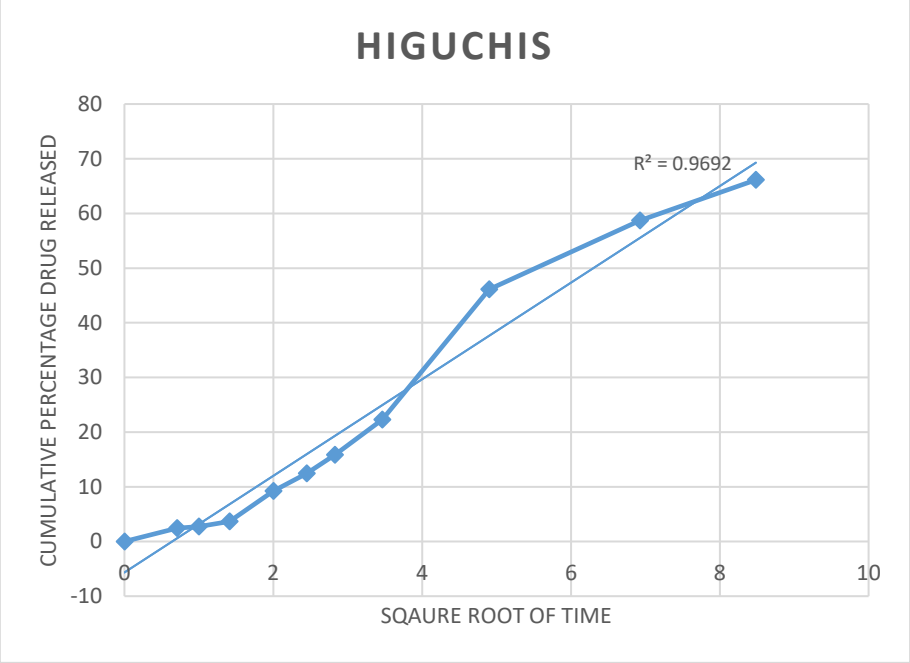
3.5.2 Release kinetics

Table 26: Drug release kinetics data of the formulation

Time (Hours)	Cumulative percentage drug released	Percentage drug remaining	The square root of time	log percentage remaining	log time	log Percentage drug released

0	0	100	0	2	0	0
0.5	2.4534	97.5465	0.7071	1.9892	0	0.3897
1	2.7001	97.2998	1	1.9881	0	0.4313
2	3.6842	96.3157	1.4142	1.9836	0.3010	0.5663
4	9.2419	90.7580	2	1.9578	0.6020	0.9657
6	12.4638	87.5361	2.4494	1.9421	0.7781	1.0956
8	15.8213	84.1786	2.8284	1.9252	0.9030	1.1992
12	22.2753	77.7246	3.4641	1.8905	1.0791	1.3478
24	46.0913	53.9086	4.8989	1.7316	1.3802	1.6636
48	58.6918	41.3081	6.9282	1.6160	1.6812	1.7685
72	66.1252	33.8747	8.4852	1.5298	1.85733	1.8203

Figure 12: Higuchi's Plot



3.5.3. Regression values for various kinetic models

Table 27: Regression values obtained for the plot of various kinetic models

Kinetic Model	Zero-order kinetics	First-order kinetics	Higuchi's Plot	Korsmeyer's-Peppas plot
Regression value	0.9078	0.9588	0.9692	0.7508

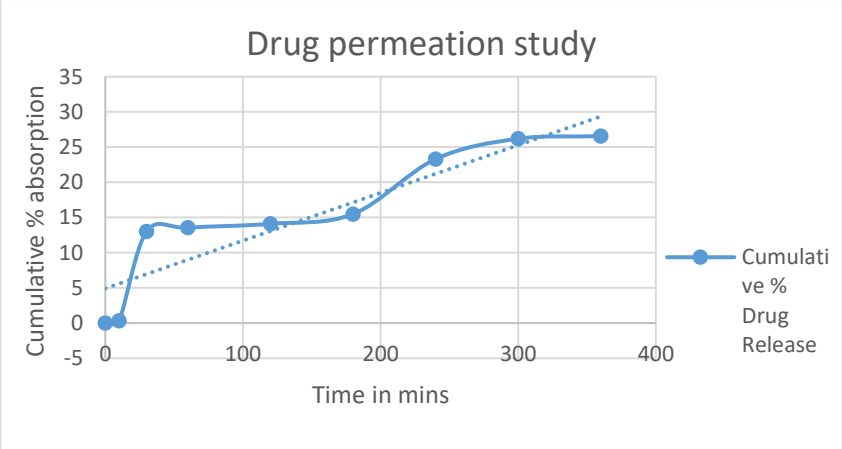
3.5.4 *Ex-vivo* Permeation Study

Ex-vivo permeation studies were conducted using goat mucosal tissues. SNF was used as a solvent medium for the studies. The study was conducted for 6 hours. 26.55% permeation was observed in 6 hours. The drug permeation obtained was higher *ex*-vivo than expected in comparison to the *in-vitro* drug release study results. This could be due to better penetration of the drug through the nasal mucosal tissue than through the dialysis membrane.

Table 28: *Ex-vivo* drug permeation study data

S.No	Time	Cumulative % Drug Release
1	0	0
2	10	0.3358
3	30	12.9918
4	60	13.5635
5	120	14.1068
6	180	15.4854
7	240	23.3055
8	300	26.1947
9	360	26.5529

Figure 13: plot of *ex-vivo* permeation study



CONCLUSION:

Based on the results it concludes that the high-speed homogenization method is successful in the formulation of NLCs with required properties. The prepared NLCs of Valproic acid releases 66.12% in 72 hours and permeations of 26.55% in 6 hours. The permeation study results indicate that the formulation is capable of getting absorbed through the nasal route. Particle size was found to be 142.5nm which is the optimum range for BBB permeation. The prepared nanoemulsion offers a promising approach for improving the pharmacokinetics of the drugs at the molecular level. Thus, the dose of drugs can be reduced without affecting its pharmacological response.

ACKNOWLEDGEMENT:

The authors would like to thank the Department of Science and Technology Fund for the improvement of science and technology infrastructure in universities and higher educational Institutions (DST-FIST), New Delhi for their Infrastructure support to our department.

REFERENCES:

1. Lopez-Munoz F, Baumeister AA, Hawkins MF, Alamo C: The role of serendipity in the discovery of the clinical effects of psychotropic drugs: beyond of the myth. Actas Esp Psiquiatr. 2012 Jan-Feb;40(1):34-42.
2. Meunier, G. Carraz, Y. Neunier, P. Eymard, and M. Aimard, "Pharmacodynamic properties of N-dipropylacetic acid," Th´erapie, vol. 18, pp. 435–438, 1963.
3. S. Lebreton, G. Carraz, H. Behriel, and H. Meunier, "Pharmacodynamic properties of 2,2-dipropylacetic Acid. III," Therapie, vol. 19, pp. 457–467, 1984.

4. S. Lebreton, G. Carraz, H. Meunier, and H. Behriel, "Pharmacodynamic properties of 2,2-dipropylacetic Acid. 2nd report on its anti-epileptic properties," Therapie, vol. 19, pp. 451–456, 1964.
5. El-Habr EA, Dubois LG, Burel-Vandenbos F, Bogeas A, Lipecka J, Turchi L, Lejeune FX, Coehlo PL, Yamaki T, Wittmann BM, Fareh M, Mahfoudhi E, Janin M, Narayanan A, Morvan-Dubois G, Schmitt C, Verreault M, Oliver L, Sharif A, Pallud J, Devaux B, Puget S, Korkolopoulou P, Varlet P, Ottolenghi C, Plo I, Moura-Neto V, Virolle T, Chneiweiss H, Junier MP: A driver role for GABA metabolism in controlling stem and proliferative cell state through GHB production in glioma. Acta Neuropathol. 2017 Apr;133(4):645-660. doi: 10.1007/s00401-016-1659-5. Epub 2016 Dec 28. [PubMed:28032215]
6. Rosenberg G: The mechanisms of action of valproate in neuropsychiatric disorders: can we see the forest for the trees? Cell Mol Life Sci. 2007 Aug;64(16):2090-103. [PubMed:17514356]
7. Vyas TK, Shahiwala A, Marathe S, Misra A. Intranasal drug delivery for brain targeting. Current drug delivery. 2005 Apr 1;2(2):165-75.
8. Khan AR, Liu M, Khan MW, Zhai G. Progress in brain targeting drug delivery system by nasal route. Journal of Controlled Release. 2017 Dec 28;268:364-89.
9. Erdő F, Bors LA, Farkas D, Bajza Á, Gizurarson S. Evaluation of intranasal delivery route of drug administration for brain targeting. Brain research bulletin. 2018 Oct 1;143:155-70.
10. Crowe TP, Greenlee MH, Kanthasamy AG, Hsu WH. Mechanism of intranasal drug delivery directly to the brain. Life sciences. 2018 Feb 15;195:44-52.
11. Mishra B, Patel BB, Tiwari S, Colloidal nanocarriers: a review on formulation technology, types and applications toward targeted drug delivery, Nanomedicine. 2010; 6: 9– 24.
12. Mǘller RH, Radtke M, Wissing SA. Nanostructured lipid matrices for improved microencapsulation of drugs. Int J Pharm 2002;242:121
13. Beck R, Guterres S, Pohlmann A. Nanocosmetics and Nanomedicines: New Approaches for Skin Care, SpringerVerlag, Berlin Heidelberg; 2011.
14. Mǘller RH, Runge SH. Solid lipid nanoparticles (SLN1) for controlled drug delivery. In: Benita S, ed. The submicron emulsion in drug targeting and delivery. Harwood Academic Publishers, the Netherlands; 1998. p. 219–34.

15. Gasco MR. Solid lipid nanospheres from warm microemulsions. Pharm Technol Eur 1997;9:52–8.
16. Cortesi R, Esposito E, Luca G, Nastruzzi C. Production of lipospheres as carriers for bioactive compounds. Biomaterials 2002;23:2283–94.
17. Kržič M, Sentjurc M, Kristl J. Improved skin oxygenation after benzyl nicotinate application in different carriers as measured by EPR oximetry *in vivo*. J Controlled Release 2001;70:203–11.
18. Mei Z, Chen H, Weng T, Yang Y, Yang X. Solid lipid nanoparticle and microemulsion for topical delivery of triptolide. Eur J Pharm Biopharm 2003;56:189–96.
19. Shrestha H, Bala R, Arora S. Lipid-based drug delivery systems. Journal of pharmaceutics. 2014;2014.
20. Bhavar S, Sarode R, Basarkar G. Formulation development and *in vitro* evaluation of *in situ* lisinopril gel for nasal administration.
21. Alsarra IA, Al-Omar M, Belal F. Valproic acid and sodium valproate: comprehensive profile. Profiles of Drug Substances, Excipients and Related Methodology. 2005 Jan 1;32:209-40.
22. Van Dooren AA, Müller BW. Purity determinations of drugs with differential scanning calorimetry (DSC)—a critical review. International journal of pharmaceutics. 1984 Jul 1;20(3):217-33.
23. Kumar, B. P., Chandiran, I. S. & Jayaveera, K. N. Formulation and evaluation of glimepiride loaded cellulose acetate microparticles. International Journal of Pharmacy and Pharmaceutical Sciences, 2014; 6: 511–515.
24. Nnamani PO, Hansen S, Windbergs M, Lehr CM. Development of artemether-loaded nanostructured lipid carrier (NLC) formulation for topical application. International journal of pharmaceutics. 2014 Dec 30;477(1-2):208-17.

AUTHORS:

(1st Author): Arun Krishnanand, M.Pharm, JSS College of Pharmacy, Ooty, The Nilgiris – 643001

(1st Author): Abhishek Dey, M.Pharm, JSS College of Pharmacy, Ooty, The Nilgiris – 643001

(2nd Author): S Kamesh, M.Pharm, JSS College of Pharmacy, Ooty, The Nilgiris – 643001

(2nd Author): Vignesh Selvaraj, M.Pharm, JSS College of Pharmacy, Ooty, The Nilgiris – 643001,

(3rd Author): Dr. Natarajan Jawahar, M.Pharm, Ph.D. , JSS College of Pharmacy, Ooty, The Nilgiris – 643001,

(Correspondence Author): Dr. Senthil Venkatachalam, Senthil.v@jssuni.edu.in, 9842650602