

Development of Capsules Filled with Phenytoin and Berberine Loaded Nanoparticles- A New Approach to Improve Anticonvulsant Efficacy

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ABSTRACT

Introduction: Currently used anticonvulsant drugs are not totally effective to control seizures. Phenytoin is a classic instance where its efficiency is inhibited through high metabolism (90%-95%) and back transport through cytochrome P450 and P-glycoprotein, respectively. To explore and attain improved anticonvulsant efficacy combination therapy with nanotechnology has been adopted in this research. **Objective:** development of nanotechnology-based drug delivery system by filling berberine and phenytoin nanoparticles in capsules. **Method:** phenytoin and berberine nanoparticles were formulated individually by utilising a solvent evaporation technique and later the mixture is filled in the hard gelatine capsule to create a single-unit dosage. **Results:** The formulated nanoparticles were evaluated in terms of FTIR and DSC studies, mean particle diameter and zeta-potential, entrapment efficiency and *in vitro* release. FTIR and DSC studies had proved that formulation components are compatible. SEM report revealed that all the nanoparticles were smooth, spherical in shape and within the size range of 100-500nm. Zeta-potential of phenytoin and berberine nanoparticles was negative (-2.61 and -35.9mV, respectively). *In vitro* release kinetics was in the accordance with the Higuchi model. The improved efficacy was proved by MES and Histopathological studies. **Conclusion:** The successfully developed Capsules filled with the Nanoparticles (Combination therapy) exhibited improved efficacy than that of single phenytoin therapy.

Key words: Status epilepticus (SE), Phenytoin, Berberine, Nanoparticles, Cytochrome P-450, P-Gp efflux transporters.

INTRODUCTION

Status epilepticus is a serious neurological disorder that results from abnormal/excessive or synchronous neuronal activity of the brain. Normally a raised concentration of calcium ion in neuronal tissue triggers a cascade of biochemical responses that leads to the death of neuronal cell following status epilepticus¹ and subsequently leads to cellular damage, disruptions and eventually to cell death.² The mortality and morbidity rate of the status epilepticus is higher due to the epileptic focal point of cerebral neurons that frequently fires and thus had raised metabolic demands. It has been found that global occurrence of active epilepsy has extended to 4-5 per 100³ whereas

WHO's report stated that, around 50 million people are affected.⁴ Since termination of seizure activities and control of brain damage are quite difficult to attain, prompt and safer efficacious therapy is needed to prevent loss of cerebral auto regulation and neuronal damage.

More than 20-40% epileptic patients are being restricted to anti-epileptic drugs.⁵ The significant issue in the most anticonvulsant drugs has been identified as the efficacy. Phenytoin is the most widely used drug in tonic clonic seizure and status epilepticus^{6,7} This drug is a hydantoin derivative under barbiturates group and its efficacy is less due to the metabolism of more than 90% of

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the drug into inactive 5-(p-hydroxyphenyl)-5-phenylhydantoin by cytochrome P450⁸ and back transportation by P-glycoprotein (P-gp) efflux transporter.⁹

The aim of this research is to explore and attain improved anticonvulsant efficiency of combination therapy through the development of nanotechnology-based drug delivery system incorporating berberine and phenytoin. Berberine is an isoquinoline alkaloid, natural COX-2 inhibitor,¹⁰ P-Gp inhibitor,¹¹ cytochrome P450 inhibitor and potent inhibitor of CYP1A and CYP3A^{12,13} and therefore it has the potential to maximize the free drug concentration by reducing the metabolism and back transportation of phenytoin. It also exerts other activities such as anti-inflammatory, anti-diabetic, lipid per-oxidation, neuro-protective activity and most importantly anticonvulsant activity.^{14,15} Subsequently the next strategy was 'adaptation of nanotechnology which positively impacts the efficacy improvement since nanotechnology is a powerful platform and most promising strategy to CNS drug delivery, by which the drugs are efficiently delivered to the brain, by crossing the blood brain barrier.¹⁶

By utilising both the strategies, the phenytoin and berberine nanoparticles were formulated (Individually) through solvent evaporation and sonication methods and they were loaded in the 1" sized hard gelatine capsules designed as a single unit dosage and later on the nanoparticles were optimized by *in vitro* release and entrapment efficiency. Since, phenytoin has low solubility, high permeability and of a class II category as per BCS classification¹⁷ the PEG 6000¹⁸ (solubility enhancer) and HPMC E15¹⁹ carrier were used to formulate phenytoin nanoparticles. Similarly, berberine has low solubility, low permeability and of class IV category and hence to enhance the permeability and solubility, sodium caprate²⁰ and PEG 6000 were used respectively. Likewise, HPMC E15 was used as an effective controlled-release carrier which has excellent biocompatibility and biodegradability to provide hydrophilic environment in drugs with intermolecular hydrogen bonding and semi inter penetrating network in the formulation.

MATERIALS AND METHODS

Methodology

Research Design

Since the current research is based on analysing and exploring the effects of drugs in laboratory experimental research design has been adapted by the researcher.

Research Approach

The research approach adapted by the researcher would be quantitative approach.

Sampling

- The components Phenytoin and Hydroxyl Propyl Methyl Cellulose E₁₅ were obtained from Medopharm, Chennai (India), at free of cost.
- Sodium Caprate and berberine chloride were purchased from Sigma Chemical Co., USA.
- PEG 6000 was procured as a sample-gift from Matrix, India.
- Methanol and Ethanol were purchased from Merck (India).
- Other reagents (Analytical grade) were purchased from SD Fine chem. (Bangalore).

The obtained samples were subjected to several studies, such as: SEM study, DSC Study and histopathological study, towards attaining the research objectives.

Pre-Formulation Study

The pre-formulation study in this research makes use of groups of studies like solubility study in which the enhancers' solubility would be studied at equilibrium; similarly, the permeability test was conducted to check the permeability enhancer's concentration optimization; etc.

Solubility Study

Three solubility enhancers were taken as variables for the test that has been adapted from the study of Higuchi and Connors.²¹ With an excess of drug (200mg), Phenytoin was added to 10mL of distilled water that has different concentration of solubility enhancers: PEG 6000, Tween 80 and SLS (0.5%, 1.0%, 1.5% and 2.0%). The shake-flask method was utilised for separation of phases, for 24 h. When equilibrium was achieved, supernatant liquid was removed and filtered through 0.22µm filter paper, which was later analyzed spectrophotometrically at 205nm, by Shimadzu UV -1800 visible spectrophotometer. The same solubility test was repeated thrice and the same techniques were carried out for berberine except it was spectrophotometrically analysed at 345nm.

Permeability Study

Egg membrane preparation

The permeability study was carried out to optimize the concentration of permeability enhancer in the formulation. Egg membrane was prepared by soaking the egg in 0.1N HCl overnight after which the egg membrane was peeled off and utilized for permeability test.²¹

This experiment was carried out in Franz diffusion cells at 37°C. The egg membrane subsequently increased among donor as well as receptor cells after it has been equilibrated with phosphate buffer 6.8. Donor cell berberine and sodium caprate in different ratios (1:0, 1:0.2,

1:0.4, 1:0.8 and 1:1) were taken separately with 6.8 phosphate buffer 2 mL and the receptor sections were filled by phosphate buffer and mixed with a layered magnetic bar. Samples were extracted from receptor sections at pre-determined time intervals and then replaced by the same quantity of phosphate buffer. The amount of permeated drug calculated according to the drug concentration in receptor medium determined spectrophotometrically at 345nm (Shimadzu UV-1800 visible Spectrophotometer).

Preparation of Nanoparticles

Preparation of Phenytoin-Loaded Nanoparticles

Phenytoin nanoparticles were prepared by solvent evaporation method.²² Required HPMC E₁₅ was weighed and dissolved in 10 ml of distilled water and later 1.25 % PEG 6000 was added with the mixture. Likewise, weighed phenytoin drug was dissolved in 5mL of ethanol and was injected into aqueous HPMC E15 solution in mechanical stirrer till it was mixed uniformly. The mixture was later on poured and entirely dried up in a flask of rotary evaporator on a rotating speed with 80 rpm with the water bath at 60°C. The obtained dry film on the walls of the rotating flask was shaken with 5mL of ethanol and the resulting suspension was subjected to sonication at 60% amplitude for 60 sec in a probe sonicator. Then, the sediment is removed by centrifugation at 10,000rpm for 15 min at 37°C and finally the sedimented phenytoin nanoparticles were dried. Formulation parameters are reported in the Table 1.

Preparation of Berberine-Loaded Nanoparticles

Berberine-loaded nanoparticles were also prepared by the same solvent evaporation method as phenytoin-loaded nanoparticles. Accurately weighed quantities of berberine were liquefied in ethanol and the solution was mixed with the aqueous solution of HPMC E15, PEG6000 and sodium caprate and magnetically stirred until the mixture was uniform. The resultant mixture was then placed in rotary evaporator at a speed of 80rpm at 60°C; which was later subjected to probe sonication for 60 sec and centrifugation at 10000rpm for 15min. finally the resultant pellet that contains berberine-loaded nanoparticles were dried. Formulation parameters are reported in the Table 2.

Nanoparticle characterisation

UV Spectroscopy (Determination of λ_{max})

Stock solution of phenytoin and berberine were prepared with methanol. Dilutions were made with methanol at the concentration of 10mcg/ml; where the UV spectrum was recorded in the range of 200-400 nm by using Shimadzu spectrophotometer.

Table 1: Preparation of Phenytoin Nanoparticles.

FORMULATION CODE	DRUG: HPMC E15	PEG6000
PNANO1	1:0.25	1.25
PNANO2	1:0.5	1.25
PNANO3	1:0.75	1.25
PNANO4	1:1.0	1.25
PNANO5	1:1.25	1.25
PNANO6	1:1.5	1.25

Table 2: Preparation of Berberine Nanoparticles.

FORMULATION CODE	DRUG: HPMC E15	PEG6000	SODIUM CAPRATE
BNANO1	1:0.25	1.5	1.0
BNANO2	1:0.5	1.5	1.0
BNANO3	1:0.75	1.5	1.0
BNANO4	1:1.0	1.5	1.0
BNANO5	1:1.25	1.5	1.0
BNANO6	1:1.5	1.5	1.0

Standard Calibration Curve of Phenytoin Sodium and Berberine

Accurately weighed phenytoin (50mg) was dissolved in 50ml methanol. Dilutions were made in the range 5, 10, 15, 20, 25 and 30 μ g/ml and maximum absorbance was monitored at 205nm²³ against the solvent blank ethanol. Similarly, berberine of 50mg was dissolved in 50mg of methanol. Dilutions were made with the concentration in the range of 5-30 μ g/ml and the absorbance was measured at 345nm.²⁴ Absorbance values of the Y-axis were plotted against the concentration in X-axis.

FTIR Analysis

FTIR studies were carried out to study the interaction between formulation components in phenytoin-loaded nanoparticles, berberine-loaded nanoparticles and nanoparticles filled capsules FTIR spectrums of phenytoin nanoparticles, berberine nanoparticles and nanoparticles filled capsules were recorded by Shimadzu Fourier Transform Infrared Spectrophotometer (Shimadzu UV 1800). Similarly, KBr disks were formed through compressing powders, at 5 tons of pressure, for 5 min in the hydraulic press. Most of the readings of spectrum were obtained in between 400 to 4000 per cm.

Differential Scanning Calorimetry (DSC)

DSC analysis was carried out using model Q-2000 Thermal Analyzer instrument. DSC cell was cleansed by dry nitrogen at 50ml per min and then the weighed sample (3-5 mg) was placed in an ordinary aluminium pan at

the temperature of 25 to 300°C with the heating rate at 10°C/min. Thermograms for phenytoin-loaded and berberine-loaded nanoparticles and nanoparticles filled capsules were recorded based on the melting point of the sample.²⁵

Computer Based Drug Interaction Study

The interaction analyses among the polymer and drug was performed in the biovia discovery studio, 2017 platform. The polymer HPMC E15 (cid: 57503849), berberine (cid: 2353) and phenytoin (cid: 1775) structures were collected from the PubChem database. Initially the grids are generated around the polymer with the coordinates of X (1.152), Y (3.542) and Z (-0.0842). Finally, both drugs are docked around the polymer and the stable complex will be saved for interaction analysis. CHARM based algorithm was used to make an interaction between the drug and polymer.

Molecular Dynamic (MD) Simulation/Interaction of Berberine Nanoparticles

Molecular dynamic study of Phenytoin nanoparticle and Berberine nanoparticles were evaluated by using BIOVIA Discovery Studio and 2017 and Materials Studio 7.0 software. In this analysis the MD simulations and docking studies were utilized towards investigating the Phenytoin/Berberine-HPMC-PEG nanoparticles formation and the interactions between PEG and also the details of Phenytoin/Berberine drugs in the molecular level.

SEM Analysis

The diluted phenytoin nanoparticles and berberine nanoparticles were separately examined under Scanning Electron Microscope (Zeiss, Model EVO 18), which was later used to examine the detailed morphological characterization. In this study, the nanoparticles were appended to a carbon tape at the top of SEM aluminium stubs and they were covered with gold by using sputter oater (Electron Microscopy Sciences of model 550X). The Phenytoin nanoparticles and Berberine nanoparticles that were gathered in the extension chamber along with the SEM images of various areas were analysed. The analysis was performed at a high vacuum of 300m Torr and the entire SEM experiment was done at an accelerating voltage of 10kv.

Evaluation of Particle Size and Zeta-Potential

Particle size and zeta-potential of both phenytoin and berberine-loaded nanoparticles were determined by Malvern Zetasizer (By laser light scattering method - Mastersizer-2000; and Malvern, UK). 1mL of the diluted nanoparticles were sonicated for 30 sec and

placed in the master size cell and later the particle size and zeta-potential were identified.

Determination of Drug Loading and Encapsulation Efficiency

Prepared phenytoin-loaded or berberine-loaded nanoparticles were centrifuged at 10,000rpm for 15 min to isolate the untrapped drugs. The amount of free drugs in supernatant was quantified spectrophotometrically. Required dilution was done with methanol and the solution was later filtered and the rate of absorbance was measured at 205 and 345nm, respectively.

In vitro Release Studies

In vitro release studies were performed in the USP II dissolution test apparatus (Labinda, Disso) with the prepared nanoparticles. In the dissolution chamber, a 900ml of dissolution media, 0.1N HCL of pH value 1.2 for 2 h and phosphate buffer of pH value 6.8 for 24 h was taken and maintained at 37°C ± 0.5°C. Samples were introverted in predetermined intervals upto 24 h and the equal amount of buffer was replaced. The samples were filtered and the dilution was carried in the concentration of 10mg/mL and then the amount of drug released was quantified spectrophotometrically.

Formulation and Evaluation of Capsules Filled with Nanoparticles

Optimized phenytoin- and berberine-loaded nanoparticles (PNANO-5 and BNANO-5) equivalent to 100 mg were filled in 1" sized hard gelatine capsule.

Weight Variation and Uniformity of Content Test

20 capsules were weighed with the help of an electronic balance and the experiment was conducted based on the USP official limits. 10 Capsules were weighed and the net contents were removed. Nanoparticles equivalent to 100mg of phenytoin and berberine were taken in separate 100mL volumetric flask, methanol was added and the absorbance was measured at 205nm and 345nm, respectively.

In vitro Release Studies

In vitro dissolution analyses were carried out for Nanoparticles filled capsules²⁶ in dissolution test apparatus (Labinda, Disso) using 900mL of dissolution media, 0.1N HCl of pH value 1.2 for 2h and phosphate buffer of pH value 6.8 for 24 h at 37±0.5°C. Samples were introverted in pre-determined intervals up to 24h. Equal amount of buffer was replaced. Then the samples were filtered and dilutions were carried on with the concentration of 10mcg/ml and the amount of drug released was quantified spectrophotometrically.

Kinetic Analysis of Drug Release Profiles

The calculated data from the drug release was fitted into Higuchi and Korsmeyer-Peppas exponential equation, to establish the mechanism of drug release.

$$Q = k t^n,$$

-Where Q is the percentage of drug release, t is time, k is a constant and n is the diffusional exponent which is the significant marker of the mechanism of drug release, from the dosage form.

Acute Toxicity Analysis

Acute toxicity analysis was conducted for Phenytoin and Berberine nanoparticles to ascertain the safe dose. Six healthy albino rats were selected for the lab test and the selected animals were addressed at Animal House Facility Institute until they attained significant weight of 150-180gm, appropriate for the current investigation. Institutional Animal Ethical Committee approval (IAEC/CHITRAKARTHIKEYINIS/AU/1524559770/Ph.D/KMCP/40/2018) was obtained for exploitation of the animals as well as the research design. Phenytoin and Berberine nanoparticles were suspended in DMSO individually and administered (5, 50, 300 and 2000 mg/kg) to the Wistar albino rat groups, in a solitary oral dose, through gavages by a feeding needle. The direct group acquired an equal amount of vehicle; observations were noticed systematically and constantly as per OECD guideline-423. The visual examinations such as mobility, skin changes, sensitivity to the pain and sound, aggressiveness and respiratory movements were systematically noticed. Finally, the numbers of survivors (sample rats) were noted down after the first 24h and later the remaining animals were monitored for 14 more days with daily-based observations and reports. The toxicological influence was estimated, on the source of mortality.²⁷

Evaluation of Anticonvulsant Activity by MES STUDY Six groups of six Wistar albino rats respectively, with 150-180g of either sex were employed for the current analysis. Group A acquired the vehicle; Group B1, B2 B3, received phenytoin 25mg/kg, berberine 25mg/kg and its free drug combination respectively as a standard; Group C1, C2, C3 received (Test samples) phenytoin-loaded nanoparticles 5mg/kg, berberine-loaded nanoparticles 5mg/kg and phenytoin and berberine-loaded nanoparticles together, respectively. Experiment was initiated after an hour of oral treatment by using either the vehicle or the nanoparticles. Equipment with pinna electrodes was employed to deliver the stimuli. Under the strength of stimulus, 45mA, 50 Hz for 0.2s, all the rats treated with vehicles revealed the features

of extensor tonus. Animals were then monitored intimately for 2 min. It was identified that the vanishing of tonic hind limb extensor was employed, as a positive criterion. Inhibition percentages of seizures related to control was also measured.²⁸

Histopathology Study

Cresylviolet Staining and Morphological Observation of Brain Regions

Different groups of albino Wistar rats were pre-treated with pure drug and combination, individual nanoparticles and combined nanoparticles (Capsule), respectively. Later on the animals were sacrificed under anaesthesia and the brain-cortex sections were taken for analysis to study the histopathological changes, where the Cresyl violet was used as a histopathologicalstainer.²⁹ Cresyl violet stained both neurons and glia and produced a brilliant violet colour as a result. Staining and Morphological observation was carried out in three steps: (i) sectioning the brain. (50 μ m in thickness); (ii) placing the brain sections on the slides and application of the stain and (iii) viewing the stained sections in conjunction with a brain atlas.

RESULTS AND DISCUSSION

Pre-Formulation Study

In the pre-formulation study, group of studies has been carried out.

Solubility Study

Solubility study was carried out with 3 different solubility enhancers such as SLS, Tween-80 and PEG-6000. Among the three, 1.25% of PEG-6000 increased the phenytoin solubility to 6-fold and 1.5% increased the berberine solubility to 7 fold times, respectively. The study established that PEG-6000 was selected as a good candidate for solubility enhancement and it was miscible with all proportions of solvent and doesn't support the microbial growth.

Permeability Study

The permeability study was carried out with the egg membrane that was clamped in the Franz diffusion cell. Egg membranes assist in optimizing the concentration of the permeability enhancer. Sodium caprate and Berberine mixture were placed in the diffusion cell and then the concentration was optimized. The results thus revealed that a threefold of drug permeation was increased at the concentration level of 1.0% due to the coupling of sodium caprate with the drug with the appropriate concentration level.

FTIR Study

Similarly, the FTIR analysis was utilized to examine the possibilities of the physicochemical interaction between the drug and the polymer.³⁰ FTIR spectrums were taken to reveal the interaction of Phenytoin, Berberine and its nanoparticles. In the FTIR spectrum, the peak at 1731.6cm^{-1} represents the carbonyl C=O stretching of the PEG and the peak at 2912.95cm^{-1} and 2848.35cm^{-1} represents the methyl and propyl group of HPMC E15. The spectrum of the nanoparticles demonstrated that no characteristic set of peaks interacted; however, it clearly proved that the drugs were encapsulated by HPMC E15 and there were no significant interactions between the drug and the polymer. (Figure 1).

DSC Study

DSC is utilized to examine any physicochemical interactions between the drug and the polymer. The rapid or drastic changes in shifts of exothermic and endothermic peaks are the sign of interactions between the drug and the polymer. Absence of the drug peak in DSC thermogram indicates the amorphous or solid state of the drug in the polymer.³¹ The Endothermic peak at 196.9°C and Exothermic peak at 81.7°C , 115.6°C and 186°C in Berberine was found to be absent in Berberine nanoparticles; similarly the characteristic set of Exothermic peak at 69.0°C and 90.0°C of Phenytoin was absent in the Phenytoin nanoparticles. Thus, it was clearly proved that the Phenytoin and Berberine drugs were incorporated in HPMC E15. (Figure 2)

Computer Based Drug Interaction

The computer-based interaction studies between the polymer and the drug projected the molecular level-insight view and the nature of the drug's stability. Results of the interaction showed that, both the drugs (Berberine and Phenytoin) interacted well with the poly-

mer. Analysis of the HPMC E15 polymer and Berberine revealed that, three interactions are formed in the complex, they are: two-hydrogen in the Dioxo ring of the Berberine formed a hydrogen bond with the Oxygen atoms of HPMC; one hydrophobic π interaction was generated between benzene ring of berberine and hydrogen atom of polymer; Similarly, the Phenytoin formed three hydrogen bond interaction with the range of $2.06 - 2.60\text{\AA}$ distance and one hydrophobic π interaction with the polymer. Thus, the results clearly proved that both the drugs formed stable complex with HPMC E15polymer (Figure 3 and 4)

MD Simulation/interaction of Phenytoin and Berberine Nanoparticles

In this work, the MD simulations and docking studies were used to investigation the Phenytoin/ Berberine- HPMC E15-PEG nanoparticle formation and the interactions between PEG and the Phenytoin/ Berberine drugs in molecular level details. The first aim of this study was the validation of this new MD simulations procedure, which was tested on two drug-polymer systems: (a) Phenytoin – HPMC E15-PEG (b) Berberine– HPMC E15-PEG. Before MD simulations, the Force field (COMPASS) was analysed for Berberine, Phenytoin, HPMC and PEG. It shows the Forcite energy of 4092113472.7683 Kcal/mol (Berberine), 232326104289041750 Kcal/ mol (Phenytoin), 131430524.73022 Kcal/mol (HPMC) and 7953.42717 Kcal/mol (PEG). Berberine-HPMC E15- PEG and Phenytoin- HPMC E15-PEG complex molecules are built in an Amorphous cell protocol to simulate the interaction within the Berberine and Phenytoin nanoparticle. (Figure 5).

The Berberine molecule forms van der Waals energy of 565.235 with HPMC and PEG molecules. The aromatic rings in the Berberine molecules form highest van

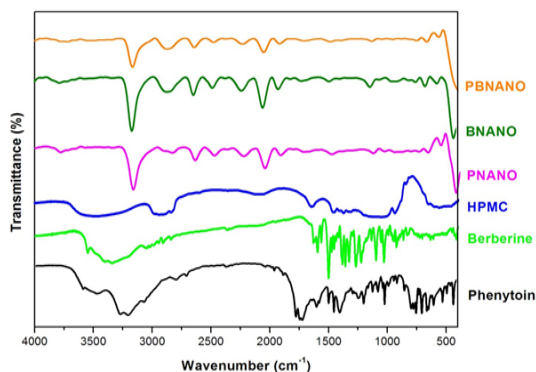


Figure 1: FTIR Spectra of: a) Phenytoin b) Berberine c) HPMC E15 d) Phenytoin nanoparticles e) Berberine nanoparticles f) Capsule filled Phenytoin and Berberine nanoparticles.

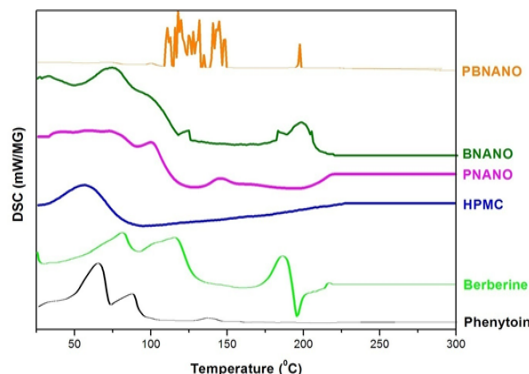


Figure 2: DSC Spectra of: a) Phenytoin b) Berberine c) HPMC E15d) Phenytoin nanoparticles e) Berberine nanoparticles f) Capsule filled Phenytoin and Berberine Nanoparticles.

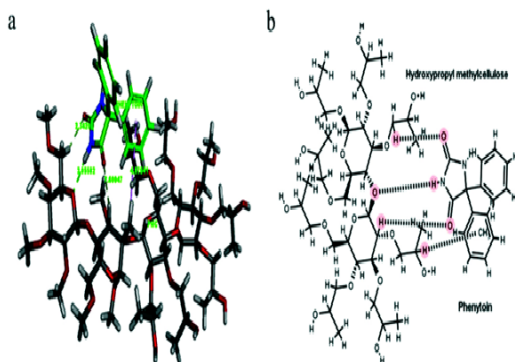


Figure 3: 3D and 2D Interaction Pattern of the HPMC E15 Polymer and Phenytoin.

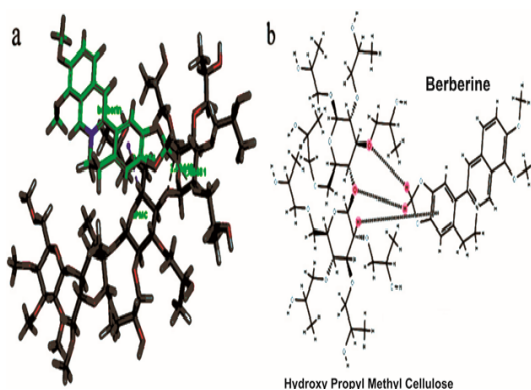


Figure 4: 3D and 2D Interaction Pattern of the HPMC E15 Polymer and Berberine.

der Waals of energy with hydrophobic groups of the HPMC E15 and PEG molecules. Similarly, the two aromatic rings of the Phenytoin molecule forms van der Waals of the energy of 716.991 with HPMC E15 and PEG molecules. During the molecular interaction analysis, the H-bond of the both HPMC E15 and PEG molecules were fixed as a stable condition to improve the Berberine and Phenytoin interactions. Additionally, the involved van der Waals interactions between the drug and polymers are accountable for nanoparticle formations. (Figure 6).

The Standard Dynamics Cascade Protocol in BIOVIA Discovery Studio shows the time-based temperature and total energy values for Berberine and Phenytoin nanoparticle compositions. From the result of molecular dynamics, the Berberine with HPMC E15 and PEG found the energy of the nanoparticle composition at the stabilized state of 136.568 Kcal mol⁻¹ and the temperature stabilized at 295 K. Similarly, Phenytoin stabilized at energy of 100 Kcal mol⁻¹ and temperature stabilized at 320 K. This results, revealed that the stability of the

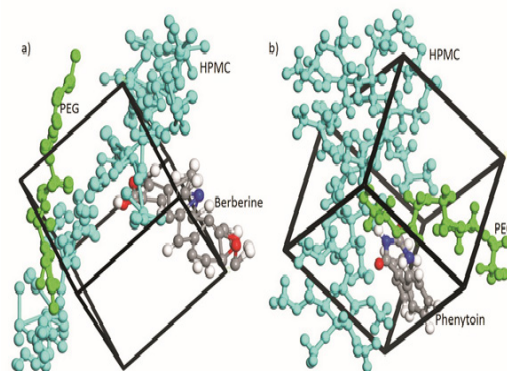


Figure 5: Formation of Molecular Interactions Between (a) Berberine nanoparticle and (b) Phenytoin nanoparticles simulated by Amorphous cell modules construction and Packing method using Material Studio Software.

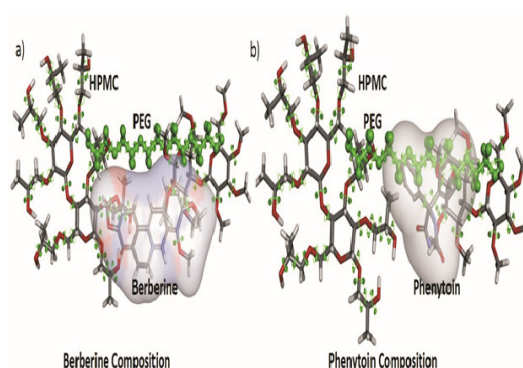


Figure 6: Van der Waals Interactions between (a) Berberine Composition and (b) Phenytoin Composition.

both Berberine and Phenytoin nanoparticles which remains unaffected by the physical condition. (Figure 7).

SEM Study

Surface Morphology was identified through the SEM photograph-study. It showed that the particles of Phenytoin and Berberine were nano-sized and they were in uniformly spherical in shape (Figure 8 and 9).

Particle Size with Zeta-Potential

Distribution of particle size by Zeta sizer proved that, all the particles were found to be smooth and uniformly spherical in shape; also it exists within the range of 100 - 500nm. Zeta-potential of Phenytoin and Berberine nanoparticles were -2.61mv and -35.9mv. The negative side of zeta-potential indicated that the system is stable. Poly Dispersity Index (PDI) of all the nanoparticles existed within the range of 1 indicated that there is no aggregation of system. (Figure 10 and 11) (Table 3).

Entrapment Efficiency and Drug Loading

In this analysis the drug was entrapped in HPMC E15, depending upon the solubility and polarity proper-

Table 3: <i>In vitro</i> Release, Drug Loading and Encapsulation Efficiency, Particle size and Poly Disperse Index for All Batches.					
Formulation code	<i>In vitro</i> release at 24 th hr	Drug Loading %	% EE	Particle size	Polydispersity index
PNANO1	84.2%	20.0	60.1	1025nm	0.702
PNANO2	90.0%	22.7	68.2	865nm	0.540
PNANO3	93.5%	25.13	75.4	745nm	0.050
PNANO4	96.4%	26.3	79.1	520nm	0.205
PNANO5	98.0%	29.46	88.4	321nm	1.000
PNANO6	97.9%	28.8	86.4	640nm	0.547
BNANO1	86.2%	17.8	62.3	985nm	0.080
BNANO2	93.5%	20.02	70.1	752nm	0.476
BNANO3	95.6%	21.48	75.2	600nm	0.520
BNANO4	97.5%	23.51	82.3	541nm	0.471
BNANO5	98.5%	25.74	90.1	471nm	0.592
BNANO6	98.1%	25.25	88.4	510nm	0.740

Results are depicted as mean ± SD (n = 3).

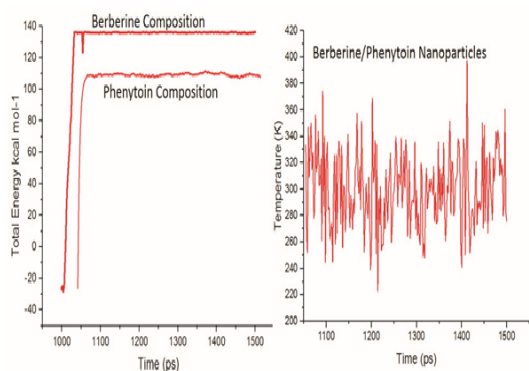


Figure 7: Time-Based Total Energy and Temperature Changes Graph in Molecular Dynamics Study.

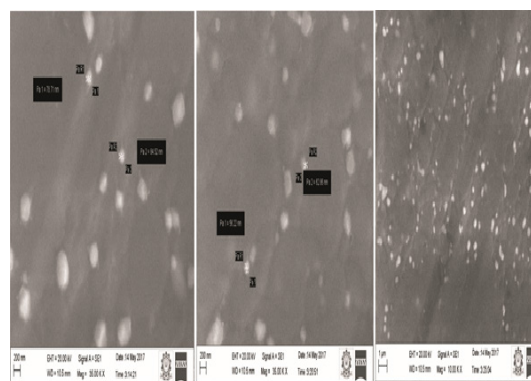


Figure 9: SEM Image of Berberine Nanoparticles.

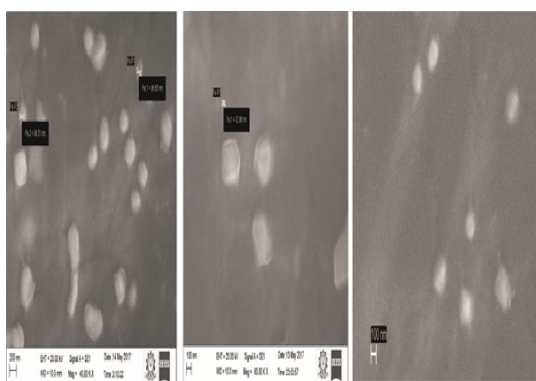


Figure 8: SEM image of Phenytoin Nanoparticles.

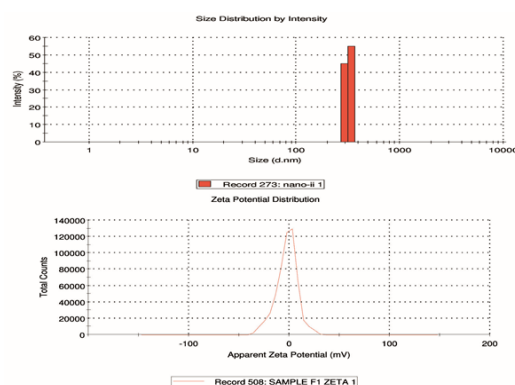


Figure 10: Particle Size and Zeta Potential of Phenytoin Nanoparticles.

ties. The gathered data shows that: if the particle size increases, the entrapment efficiency decreases, respectively; similarly, the drug loading and entrapment efficiency for PNANO-5 was estimated as 29.4% and 88.4% and BNANO-5 was estimated as 25.74% and 90.1%, respectively. Thus, the results showed a good electrostatic interaction between the drug and the polymer. Particulars of the Drug Loading and Entrapment Efficiency of all the tested batches have been tabulated (Table 3) for further references.

In vitro Dissolution Study

The dissolution analysis was performed with 0.1N HCL of pH value of 1.2 for 2h and phosphate buffer of pH value 6.8 for 24h. Dissolution profile showed that PNANO-5 and BNANO-5 were the optimized formulation and the percentage drug release of PNANO-5 was 98% whereas the BNANO-5 was 98.5%. Increased concentration of HPMC E15 releases the drug, slowly and extended the release up-to 24h. [Table 3].

Evaluation of Capsules

Various tests and studies were done and the capsules were evaluated under certain norms.

Weight Variation Test and Uniformity of Content

All the capsules were within the limit of official standard as per I.P.

In vitro Dissolution of the Prepared Capsules

The dissolution profile of Phenytoin and Berberine nanoparticles showed 98.0% and 98.5% drug release respectively and later the release was extended up-to 24 h. Thus, the release data showed that the percentage of the drug release was increased for nanoparticles when it is compared to that of pure drug. The obtained result demonstrated that the release was modified and controlled and it could be attributed by the size of nanoparticles and in the increase of surface area between the solvent and the drug surface.

Kinetics of Drug Release

To determine the drug release, different kinetic models (Such as, zero order, first order, etc) were adapted from the study of Higuchi and also the Korsmeyer-Peppas equations were obtained too. The percent release was plotted against time to get zero order plots similarly the log percent remaining was plotted against time to get first order plot; additionally, the percent release was plotted against square-root of time to get the Higuchi plot. Finally, the log cumulative % drug release was plotted against log time in hours, towards attaining the Korsmeyer-Peppas plot.

Optimized Formulations (PNANO-5 and BNANO-5) has highest regression coefficients which are 0.975 and 0.976, towards Higuchi's model indicating Fickian diffusion. The diffusion mechanism was established by fitting the data into Korsmeyer-Peppas equation and thus the regression coefficients of Korsmeyer-Peppas plot of optimized formulations are estimated at 0.892 and 0.898. The analysis showed good linearity and the diffusion was identified as the predominant mechanism of the drug release. The values of the release exponent (n) of PNANO-5 and BNANO-5 formulations are of 0.351 and 0.354, respectively and the results indicated that n -value is around 0.5. Henceforth, it was proved that the drug release follows Fickian diffusion (Figure 12).

Pharmacological Evaluation

Acute Toxicity

Once the drugs were successfully administrated in the selected animals, they were monitored and observed for 14 consecutive days and all examinations were recorded systematically and individual records were preserved for each animal, respectively. Variations of fur, skin, respiratory circulatory, eyes and mucous membranes, central and autonomic nervous systems, behaviour pattern and somato-motor activity were monitored and recorded. In this acute toxicity analysis, Phenytoin and Berberine nanoparticles were established to be toxic at 1000mg/kg and whereas to be secure up to 5mg/kg, in wistar albino rats.

MES study

The Tonic hind limb extension (THLE) occurrence was a positive parameter for MES study. THLE abolition was obtained as a safeguard technique against the MES seizures and THLE incidence along with the time of THLE in seconds were also observed for better understanding. The Statistical Analysis was conducted through "Graph Pad Prism 5" technique (San Diego,

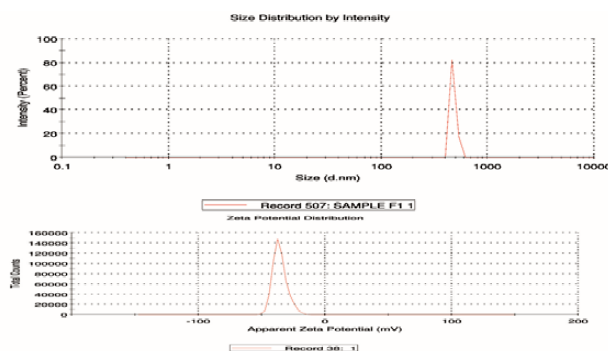


Figure 11: Particle size and Zeta Potential of Berberine Nanoparticles.

California, USA). On other-hand One-Way ANOVA was employed with post-hoc Turkey's experiment, for comparison among the groups. The $P < 0.05$ was assumed as important.

THLE arrival of action and time of action along with the test drugs such as Phenytoin nanoparticles, Berberine nanoparticles, combination of free drug and Capsules (Phenytoin and berberine combination) were recorded for standardization. The results showed that, individual nanoparticles have significant efficacy than pure drug by reducing THLE duration ($P < 0.001$). Free Phenytoin and Berberine showed slightly higher efficacy than individual nanoparticles; but Phenytoin and Berberine nanoparticles Combination was found to be more efficacious than free drugs combination and inhibit the convulsion remarkable level. Thus, the results and reports proved that efficacy has been improved with the prepared capsules. The arrival and time of THLE (Tonic Hind Limb Extension) and inhibition percentage of convulsions were noted. [Table 4] (Figure 13).

Where Mean value \pm SD ($n=6$). Statistical significance was determined by ANOVA followed by Dunnett's test, where (THLE=Tonic hind limb extension) * $P < 0.05$.

Histopathological Study

In Control cortex regions, the test rats showed decrease in neuronal cells and increase in chromatolysis and pyknotic nuclei. It has been observed through the analysis that Phenytoin 25mg/kg (i.p.) and Berberine 25mg/kg (i.p.) significantly decreased the neuronal cell death, brain cell oedema, chromatolysis and pyknotic nuclei in each and every region when compared against the control rats' cortex regions. Similarly, the rats that are treated with individual nanoparticles showed significant decrease in cell edema and neuronal cell degeneration in each and every region when compared to the control rats. Thus, the rats treated with the combination of Phenytoin and Berberine nanoparticles showed significant decrease in neuronal cell death with mild edematous nuclei in all regions as compared with combination of free drugs. (Figure 14).

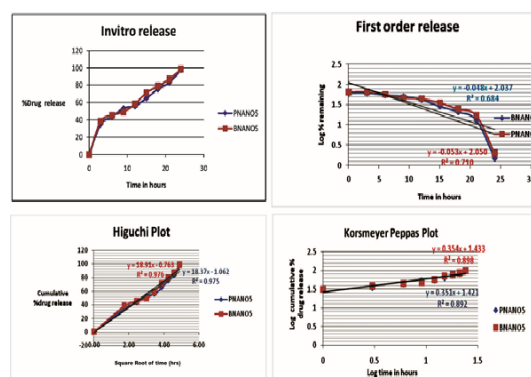


Figure 12: Kinetics of Release.

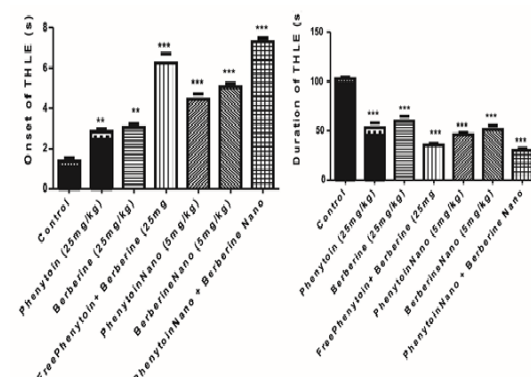


Figure 13: Onset and Duration of Convulsion Estimation by MES Study.

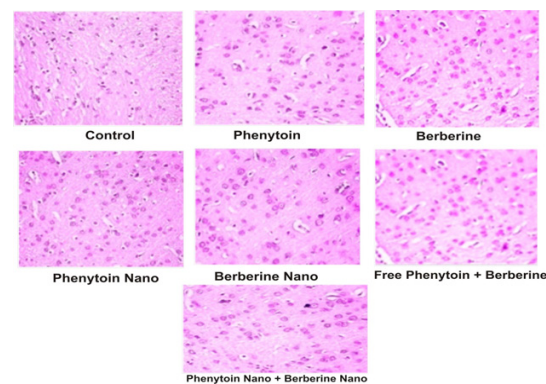


Figure 14: Histopathological Images for Pure Drug, Plain Nano Particles and Combinations of Free Drug and Nanoparticles.

Treatment	Onset of THLE(s)	Duration of THLE (s)	Percentage of inhibitions of Convulsions
Control	1.90 \pm 0.05	101.0 \pm 4.58	-
Phenytoin (25mg/kg)	2.73 \pm 0.29	57.67 \pm 3.84***	43.33%
Berberine (25mg/kg)	3.26 \pm 0.27*	68.33 \pm 2.02***	32.67%
Free Phenytoin + Berberine (25mg/kg)	6.82 \pm 0.40***	42.27 \pm 2.64***	57.3%
Phenytoin Nano (5mg/kg)	4.30 \pm 0.26***	51.00 \pm 3.21***	50.0%
Berberine Nano (5mg/kg)	5.40 \pm 0.40***	57.33 \pm 2.72***	43.67%
Phenytoin Nano + Berberine Nano 5mg/kg	7.26 \pm 0.29***	33.67 \pm 3.48***	67.33%

CONCLUSION

In this research great effort has been taken to improve the efficacy of Phenytoin anticonvulsant activity. In order to accomplish the goal, controlled release individual Phenytoin and Berberine nanoparticles were prepared and filled in with capsules to make them a single-unit dosage form. MES study thus proved that combination therapy in capsule is significantly efficacious than Phenytoin mono-therapy and its 24h drug release design thus makes simple administration and reduces the dosage frequency. The research thus argues and proves that the developed capsules are more beneficial in Status Epilepsy than individual Phenytoin therapy.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ABBREVIATIONS

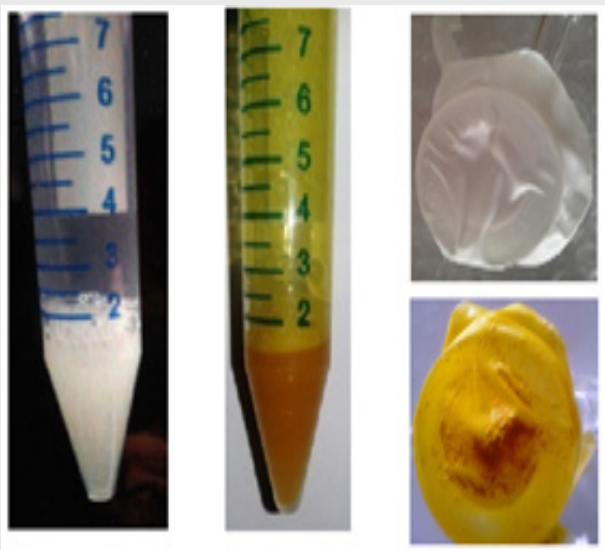
THLE: Tonic Hind Limb Extension; **MES study:** Maximum Electrical Shock study; **P-gp:** P-glycoprotein; **CNS:** Central Nervous system; **PEG:** Poly Ethylene Glycol; **CYP:** Cytochrome P-450.

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PICTORIAL ABSTRACT



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SUMMARY

- Most of anticonvulsant drugs are not explore its total efficacy. Phenytoin is a classic example and its efficacy is inhibited by high metabolism (90%-95%) and back transportation through cytochrome P450 and P-glycoprotein, respectively. So new approach has been carried out to improve the efficacy of phenytoin by adopting nano technology and combination therapy. Berberine is a co-drug and has an anticonvulsant activity. It also act as a Cox-2 inhibitor, cytochrome P-450 inhibitor which helps to maximize the free drug (phenytoin) concentration in plasma by reducing metabolism and back transportation. On account of this Phenytoin and berberine nanoparticles were prepared individually and filled in hard gelatine capsule to make as a single unit dosage form. Characterization study revealed that formulations were in nano level. Pharmacological investigation such as MES and Histo-pathological study clearly proved that combination therapy in capsule has improved efficacy than single phenytoin therapy. Hence it is more beneficial in status epilepticus than individual phenytoin.

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