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Optimization of a Phenytoin-loaded Nanoemulsion Formulation for Parenteral Use in the Treatment of Epilepsy

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Phenytoin is a classic antiepileptic drug, which exhibits potent therapeutic efficacy in controlling seizure attacks, thus signifying its importance in a basic healthcare system. However, due to the poor solubility and bioavailability of phenytoin, a large dosage had to be given orally to reach therapeutic concentration with numerous side effects. In this research, the formulation of phenytoin-loaded nanoemulsion was developed as a parenteral application for epilepsy treatment in order to control seizure attacks. The formulation was optimized by a mixture experimental design (MED) and the effects of isopropyl myristate, surfactants, glycerol, and water on the droplet size of the nanoemulsion were determined. The optimum formulation with desirable criteria for the parenteral application was obtained. The predicted response value showed no significant difference from the actual value derived from the experiment with a residual standard error (RSE) of less than 2.00 %. The drug release study showed that 50 % of the drug was released within 3 h of dissolution and the highest cumulative percentage release of phenytoin was recorded at 97 % at 6 h. The sustained release of the drug could be due to its affinity to the oil phase as phenytoin was shown to be highly soluble in the mixture of Tween 80 and Tween 85. The use of surfactants hinders the release of the drug due to its ability to form strong interfacial films surrounding the oil droplets, which can prevent the drastic release of the drug in a short period of time. Thus, the optimized nanoemulsion containing phenytoin has the potential to be used for parenteral treatment for epilepsy.

Keywords: Phenytoin; antiepileptic drug; nanoemulsion; mixture experimental design

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Epilepsy is a chronic brain disease due to abnormal electrical activity within the brain affecting either a specific area or the entire brain, resulting in generalized or partial seizures [1]. As serious as it can be, several antiepileptic drugs (AEDs) have been introduced over the years ever since the first antiepileptic drug, bromide (potassium bromide), to phenobarbital which is still in use in the modern world. Antiepileptic drugs were successful in suppressing seizures in the majority of patients, as many as 60-70% [2]. However, in 20-30% of patients, epilepsy is drug-resistant, in other words, epilepsy cases in some patients are uncontrollable. Patients with drug-resistant epilepsy will have serious health problems such as injuries, depression, anxiety, and to the highest degree, mortality [3]. The purpose of antiepileptic drugs is to control seizures as quickly as possible with minimal side effects. However, several hypotheses have been postulated as to why antiepileptic drugs fail to suppress seizures. Including the disease itself is too severe, incorrect administration of the drugs, and finally the hypotheses which researchers believe to be the key to treating epilepsy are that drugs administrated are not delivered efficiently to the target site due to the blood-brain barrier [4].

The blood-brain barrier (BBB) is a dynamic barrier protecting the brain against invading organisms, and unwanted substances, and impeding drug transport into the brain via blood circulation. Therefore, many central nervous system (CNS) diseases remained undertreated due to the inability of many therapeutic molecules to cross the BBB [5]. Phenytoin or 5, 5-diphenylhydantoin is a classic AED synthesized in 1908 before the discovery of its antiepileptic efficacy [6]. Phenytoin prevents seizure attacks by binding to the inactivated state of the voltage-activated sodium channel reducing the high influx of sodium ions which causes rapid firing of impulses leading to uncontrollable movements of the arms and legs [7]. The phenyl group at position 5 of the phenytoin structure (Figure 1(a)) is the functional group responsible for binding to the sodium-gated channel and inhibiting the continuous

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influx of sodium ions [8]. Phenytoin has been classified as a third-line agent for epilepsy treatment because of its poor solubility resulting in numerous side effects such as hypersensitivity, depression and dizziness despite being a more potent anticonvulsant compared to modern AEDs such as gabapentin [9.10].

Therefore, the utilization of nanocarriers such as nanoemulsions should be emphasized to maximize the diffusion of hydrophobic and lipophilic phenytoin across the BBB into the CNS [11]. This could bypass molecular efflux pumps through the transcellular lipophilic pathway, leading to an increase in therapeutic efficacy and a decrease in side effects without compromising efficacy. Based on these properties scientists have identified nanoemulsion as a potential drug delivery system. The advantages of using nano-emulsions as nano-drug carriers include efficient drug delivery and rapid drug penetration due to their large surface area, high loading capacity for poorly water-soluble drugs, enhanced bioavailability of lipophilic drugs and reduced drug toxicity [12-15]. Diazepam is among the first generation of drugs that were incorporated into O/W emulsions using the high-energy method. This formulation helped to reduce pain on injection to only 0.4 % of patients and showed sedation strength such as Valium.

Carbamazepine, an antiepileptic drug, was developed in an O/W nanoemulsion stabilized by 1-O-decylglycerol which showed significantly higher tissue levels and availability of carbamazepine [16]. Kelmann *et al.* [17] reported the formulation of carbamazepine O/W nanoemulsions by using spontaneous emulsification technique for parenteral treatment. According to the British Pharmacopoeia in 2009, parenteral preparations administered intravenously (IV) must isotonic and miscible with blood, have an appropriate pH value, have low toxicity and local irritation, and the active ingredient must be retained in an aqueous condition without precipitating. Therefore, the O/W nanoemulsion is the most suitable system for encapsulating the active ingredient for administration through the IV route as the bulk or continuous phase is water. The oil droplet carrying phenytoin would facilitate penetration through the BBB as the barrier is said to be a lipophilic membrane [1].

Thus, this study aimed to optimize a phenytoin-loaded nanoemulsion formulation containing with respect to droplet size using a mixed experimental design. The droplet size of less than 200 nm has the potential to be used as a parenteral application for the treatment of epilepsy. The physicochemical characteristics of the optimized nanoemulsion, the cytotoxicity of the optimized nanoemulsion, and the drug release study were then evaluated.

MATERIALS AND METHODS

Materials

Isopropyl myristate (IPM) and polysorbate 85 (Tween 85) were purchased from Merck Millipore, USA. Glycerol was purchased from JT Baker, USA. Polysorbate 80 (Tween 80) was obtained from Fluka, Sigma–Aldrich Chemie GmbH, Germany. Phenytoin was purchased from Alfa Aesar, USA. Water was treated using arium® pro ASTM Type 1 ultra-pure water system, Sartorius, Germany.

Preparation of Blank Nanoemulsion

The blank nanoemulsion (NE) (without phenytoin) was prepared by both low- and high-energy emulsification methods. The oil phase consisted of IPM and mixed surfactants, Tween 80 and Tween 85. The oil phase was mixed by magnetic stirring at 600 rpm for 1 h. The aqueous phase was mixed by magnetic stirring and then slowly added into the oil phase and mixed at 1000 rpm for 3 h at room temperature to form a coarse emulsion. The coarse emulsion was then mixed with an Ultra-Turrax T25 at 7000 rpm for 10 min under high shear.

Preparation of Phenytoin-loaded Nanoemulsion

The incorporation of phenytoin into nanoemulsion was carried out by employing a method previously described by Santos et al. [18] with slight modification. 1 ml of 0.5 M sodium hydroxide (NaOH) was added to the blank NE. The pH of the solution was 8.3. Phenytoin (0.1 % w/w) was then solubilized into the alkaline solution and homogenized using a magnetic stirrer at 600 rpm for 15 mins. The phenytoin-loaded NE was then neutralized with equal amounts of 0.5 M hydro-chloric acid (HCl). The pH of the phenytoin-loaded nanoemulsion was then adjusted to 10.00 to retain phenytoin in the nanoemulsion.

Mixture Experimental Design

Compositional optimization was carried out using a mixture experimental design (MED). The main characteristic of a given mixture is that the sum of all its constituents' proportions must be equal to 1. Theoretically, the response variable should only be influenced by the relative proportions of the components instead of the mixture volume. The D-optimal design was chosen to model the effects of four components on the droplet size of the nanoemulsion. Significant information regarding the effects of components could be interpreted through mathematical modeling by D-optimal design, hence an optimum composition can be obtained which gives the desired response value, the small droplet size of the nanoemulsion.

Table 1: Restrictions of component composition.

Variables	Range (% w/w)	
	Lower	Higher
IPM (A)	3.00	6.00
Tween 80/ Tween 85 (B)	1.50	3.00
Glycerol (C)	4.00	7.00
Water (D)	84.00	91.50

Note: IPM, Isopropyl myristate.
*The composition of phenytoin was kept constant (0.01 % w/w)

The effects of four components on the size of the nanoemulsion were evaluated according to the method used by Masoumi *et al.* [19]. The components were selected based on the solubility of the drug. Based on the results (data not shown here), phenytoin completely dissolved in Tween 85 and Tween 80, no drug precipitation was observed after 30 days. For parenteral administration, formulated nanoemulsion should have similar viscosity to that of water which is around 1 cP to avoid irritation during administration. Incorporating a viscous oil into a formulation will cause viscosity to increase therefore a less viscous oil is much more preferable for formulating parenteral nanoemulsion. Based on these criteria, IPM was selected as the oil phase. The constraints for each component were identified and selected as in Table 1.

The sum of the component's ratio must be equal to 100%: A + B + C + D = 100 %. Because of these constraints, the factor space is a convex polyhedron [20]. Constraints were applied to components based on permitted amounts used in commercial parenteral formulations. To construct a final model for optimization purposes, a non-linear response function is to be expected. Thus, a quadratic model was chosen to analyze and interpret data using the equation shown in Eq. 1.

$$y_i = \sum_{i=1}^{5} \beta_i x_i + \sum_{i=0}^{4} \sum_{j \neq i}^{5} \beta_{ij} x_i x_j + \varepsilon \qquad \text{(Eq. 1)}$$

The coefficients βi and βij represent the regression coefficients calculated from the experimental data by multiple regression. The generated model contained quadratic terms that explained the non-linear nature of the responses and multiple factor terms explaining interaction effects between factors. The generated model contained quadratic terms that explained the non-linear nature of the responses and multiple factor terms explaining interaction effects between the factors [19].

Statistical Analysis

Statistical analysis was calculated using Design-Expert software, version 7.0 (Stat-Ease Inc., Minneapolis, USA) and Statistica, version 8 (Statsoft Inc., Tulsa, Okla, USA). The quadratic model was tested for goodness-of-fit of the determination correlation (R^2). The lack-of-fit test was verified according to the probability value (P-value). The model selected must be an insignificant lack-of-fit, i.e. $\alpha \geq 0.05$ to ensure the adequacy of the selected quadratic model in its predictability and optimization capabilities. The importance of the selected variables was determined according to their probability value, with terms being significant if the probability P $\geq$ 95% ($\alpha \leq 0.05$).

Physicochemical Characterisation of Phenytoin-loaded Nanoemulsion Droplet size, Polydispersity Index and Zeta Potential Measurement

The dynamic light scattering (DLS) technique was used to determine the droplet size distribution, mean droplet size and polydispersity index (PDI) of phenytoin-loaded NE as this technique allows detection and quantification of distribution profile of small droplet sizes down to 1 nm. For sample preparation, a drop of the NE was diluted with 10 ml of water, drawn into a syringe and injected into the capillary cell coupled with an electrode. The cell was made free of air bubbles before being placed in the sample compartment of the DLS instrument (Zetasizer Nano ZS, Malvern Instrument, Malvern, UK). Samples were illuminated by a laser beam and a scattered beam of different intensities and then were detected by a photon detector at an angle of 90°. The laser beams are scattered due to the Brownian motion of the droplets in NE. The zeta potential is measured through the cell electrode by electrophoretic light scattering using the same instrument. All measurements were performed at 25°C in triplicate.

Morphology

The morphology of NE was determined by using Transmission Electron Microscopy (TEM). Samples were dropped on a copper grid supported with Formvar films and stained with uranyl acetate solution (2%). Excess uranyl acetate on the copper grid was gently wiped off using filter paper and treated under UV light for 2 mins. The samples were then viewed under TEM at different magnification levels.

Viscosity

The viscosity of the nanoemulsion was measured by Brookfield DV II+ Pro Viscometer (MA 02346, USA). 1.00 mL of the emulsion adjusted to 25.0 ± 0.1°C was

transferred into the stationary plate. The torque measuring system, which consists of a calibrated beryllium-copper spring connecting the drive mechanism to a rotating cone, measures the resistance to rotation caused by the presence of the nanoemulsion between the cone and the stationary flat plate. The resistance to rotation of the cone produces a torque proportional to the shear stress in the fluid. The shear stress was converted to absolute centipoise units (cP) from pre-calculated range charts.

pH Measurement

The pH of the nanoemulsion was measured using the pH meter (Mettler Toledo, Japan). Calibration of the pH meter was carried out using different pH buffers: pH 4.00, pH 7.00 and pH 10.00. Analysis of the nano-emulsion was then carried out in triplicate.

Encapsulation Efficiency

The entrapment efficiency of the phenytoin-loaded NE was determined using a Centrisart tube (molecular weight cut-off 10,000 Da; Sartorius, AG, Germany). The nanoemulsion was filled in the outer chamber and centrifuged at 3500 rpm for 15 min. The oil droplets containing phenytoin remained in the outer chamber while the aqueous phase moved to the sample recovery chamber through the filter. The concentration of PHT in the aqueous phase was measured using HPLC. The formula used to calculate the entrapment percentage is shown in Eq. 2.

$$\text{Encapsulation efficiency} = \frac{W_{initial} - W_{obtained}}{W_{initial}} \times 100\ \% \quad \text{(Eq. 2)}$$

Where $W_{initial}$ is the amount of phenytoin present initially and $W_{obtained}$ is the concentration of phenytoin in the aqueous phase.

Stability Studies

Freshly prepared NE was stored in the refrigerator at 4 ± 2°C and at room temperature for three months. Changes in droplet size, PDI, and zeta potential were monitored every 30 days. Accelerated stability studies were performed by centrifuging the samples at 4500 rpm for 15 mins. Samples were observed for any phase separation or precipitation formed.

Cytotoxicity Analysis

The cytotoxicity analysis was carried out on the Vero cell line by MTT assay to assess cell viability by intracellular tetrazolium reduction. The Vero cell line was maintained in DMEM medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 atmosphere. The Vero cells were seeded at $1x10^5$ cells/mL and allowed to attach for 24 h after which the cells were incubated with various concentrations of phenytoin-loaded nanoemulsion. The MTT assay depends on the cleavage of the yellow tetrazolium salt into the purple formazan crystals by active metabolic cells. The pyridine nucleotide cofactors NADH and NADPH are involved in this cellular degradation. The formazan crystals formed were solubilized by DMSO and consequential colored elucidation was quantified using an ELISA reader at a wavelength of 570 nm. Values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely, a higher absorbance rate indicates an increase in cell proliferation. The cytotoxicity was then recorded as the drug concentration that caused 50 % growth inhibition of tumor cells (IC_{50} value) using Eq. 3.

$$\text{Cell viability} = \frac{\text{absorbance sample (mean)}}{\text{absorbance control (mean)}} \times 100\ \% \quad \text{(Eq. 3)}$$

After the determination of the percentage of cell viability, graphs were plotted with the percentage of cell viability against their respective concentrations.

***In-vitro* Drug Release Study**

The in-vitro release profile of phenytoin from the nanoemulsion was evaluated by dialysis using a dialysis bag (12,000 MW cut-off; Sigma-Aldrich). The dialysis bag was hydrated in phosphate buffer solution for 12 h before use. 2 mL of the nanoemulsion dispersed in 1 mL of phosphate buffer solution (PBS; pH 6.8) was placed in the dialysis bag with both sides of the bag were sealed. The dialysis bag was then placed in a beaker containing 150 mL of phosphate buffer solution and the temperature was maintained at 37.0°C ± 0.5 while stirring was maintained at 100 rpm with a magnetic stirrer. Aliquots of the release medium (1 mL) were taken every 60 min and replaced with fresh buffer solution to maintain a constant volume. The cumulative percentage release of phenytoin was quantified using HPLC (Waters Alliance e2695, USA) equipped with a reversed phase C18 column (Symmetry C18 column – 3.5 µm, 4.6 x 75 mm) and a photodiode array detector. The mobile phase was a mixture of methanol and water (70:30) with a flow rate of 1.0 mL/min. The column temperature was set to 25°C. All samples were diluted and passed through a 0.22 µm filter before HPLC analysis.

RESULTS AND DISCUSSION

The Solubility of Phenytoin (PHT) and Encapsulation of Phenytoin in Blank Nanoemulsion

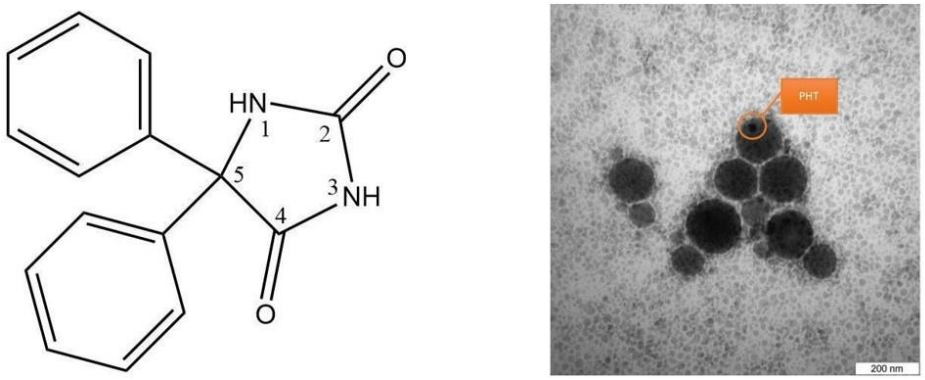
The stability and solubility of drugs in solution rely mainly on the acid dissociation constant (pKa) and partition coefficient (Log P) values [21]. Phenytoin is a weak acid with a high pKa value of 8.33 and the low Log P value of 2.47 [22]. Generally, the higher the pKa value, the weaker the acid, and the more difficult it is for drugs to be ionized thus reflecting the poor solubility of phenytoin in an aqueous solution. The reported log P value of phenytoin is the optimal value for the permeation of the blood-brain barrier (BBB) [23]. In other words, phenytoin shows high permeability

toward lipid membranes. However, it should be noted that high permeability for lipid membranes does not necessarily guarantee high solubility in oils based on the solubility test conducted in this study. Screening of various oil mixtures and surfactant mixtures showed that phenytoin was not soluble in the selected oils and surfactants. This could be due to the log P value and another factor that could affect solubility was the chemical structure of phenytoin.

Figure 1(a) shows the structure of phenytoin. The amine groups at positions 1 and 3 were acidic due to the presence of two strong adjacent electron-withdrawing carbonyl groups. However, the amino group at position 3 is subject to the electron-withdrawing effects of two carbonyl groups as opposed to the amino group at position 1 which is under the influence of only one carbonyl group. The probability of a hydrogen atom at position 1 being deprotonated or forming a hydrogen bond is very low due to the lower electron-withdrawing effect compared to a hydrogen atom at position 3 and steric hindrance by the adjacent benzene ring. Therefore, the hydrogen atom of the amine group at position 3 can be more easily to deprotonate and form the ionized phenytoin which is one of the aims of our research, to ionize phenytoin so that it could be incorporated into the nanocarrier [22].

Besides that, the high pKa value of phenytoin 8.33 is a major problem for parenteral administration. Drugs may precipitate or recrystallize if the pH of the environment is lower than their pKa, whereas the pH in our blood vein was 7.3. Due to that, the pH of the marketed parenteral phenytoin had to be adjusted to 12 to ensure complete solubility and stability of phenytoin in the solution.

Figure 1(b) showed that the addition of phenytoin into the blank nanoemulsion facilitates the ionization of the drug, allowing phenytoin to be attached to the surface of the micelle. NaOH was neutralized with equal amounts of HCl to maintain electrical neutrality, and prevent aggregation of the micelles. The NaCl produced during neutralization helps to maintain the isotonic condition required for parenteral formulation. The final pH of the phenytoin-loaded NE had to be adjusted to 10 (higher than its pKa value) because attempts to maintain phenytoin in solution at a neutral pH of 6.9 resulted in recrystallization of phenytoin after two weeks consistent with the pH solubility profile of the drug thus highlighting the difficulty of phenytoin to be formulated in parenteral solutions.



(a) (b)

Figure 1. (a) Chemical structure of phenytoin; (b) TEM of encapsulated phenytoin.

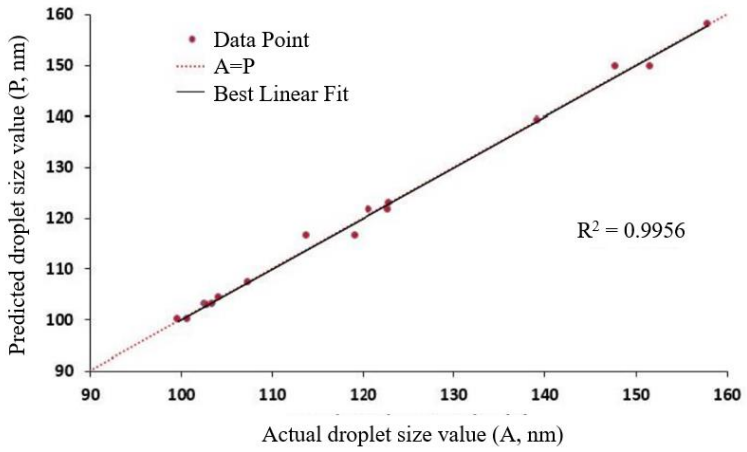


Figure 2. Correlation between mixture components.

Table 2. Predicted and actual values of droplet size of nanoemulsions obtained from D-optimal mixture experimental design.

Run No.	IPM (A)	Tween 80/Tween 85 (B)	Glycerol (C)	Water (D)	Droplet size (nm)	
					Actual	Predicted
1	5.94	1.50	4.00	88.56	151.56	149.84
2	6.00	2.46	5.47	86.07	122.87	122.48
3	3.29	2.04	7.00	87.67	100.67	100.38
4	6.00	2.00	7.00	85.00	139.30	139.57
5	3.15	3.00	5.70	88.15	102.63	102.85
6	3.00	1.50	4.00	91.50	120.71	121.87
7	3.15	3.00	5.70	88.15	103.35	102.85
8	4.88	2.94	5.35	86.83	102.81	105.58
9	5.94	1.50	4.00	88.56	147.77	149.84
10	4.58	1.50	6.09	87.83	157.90	157.89
11	3.29	2.04	7.00	87.67	99.58	100.38
12	6.00	3.00	4.03	86.97	113.79	116.82
13	3.00	1.50	4.00	91.50	122.79	121.87
14	3.97	2.39	4.00	89.64	104.21	104.03
15	4.64	2.69	4.63	88.04	107.41	105.75
16	6.00	3.00	4.03	86.97	119.19	116.82
17	5.94	1.50	4.00	88.56	151.56	149.84
18	6.00	2.46	5.47	86.07	122.87	122.48
19	3.29	2.04	7.00	87.67	100.67	100.38

Note: IPM, Isopropyl myristate.
*The composition of phenytoin was kept constant (0.01 % w/w)

Mixture Experimental Design

A design Expert was used to generate a model to fit the experiment. Based on the software, the most suitable model for prediction and optimization is the quadratic model. The quadratic model was able to accurately predict the validation set with a high R^2 (>0.90) and a low standard deviation. The quadratic model generated to predict the droplet size of the nanoemulsion can be expressed as Eq. 4.

$$y = 336.64(A) + 1281.89(B) – 26.19(C) + 121.74(D) – 2145.72(AB) + 21.75(AC) – 236.21(AD) – 2064.22(BC) – 1423.14(BD) + 317.5(CD) \quad \text{(Eq. 4)}$$

Where, A, B, C, and D represent the fractions of IPM, Tween 80/Tween 85, glycerol, and water, respectively. Based on Table 2, the lowest droplet size was observed at run number 2 and 11 with the droplet size of 100.67 nm and 99.58 nm, respectively. Figure 2 showed that the quadratic model generated was able to represent the correlation between the mixture components with an R^2 of 0.9956.

Analysis of Variance

Table 3 showed that the size of the nanoemulsion was well fitted to the quadratic model with F-value and P-value of 151.56 and < 0.0001, respectively. Based on the results, response (droplet size) was shown to be affected by components A, B, C and D.

Table 3. Analysis of variance of the fitted quadratic model for droplet size of nanoemulsion.

Source	Droplet size		
	Mean square	*F* -value	*P*-value
Model	631.38	151.56	<0.0001
Linear Mixture	1487.71	357.11	<0.0001
AB	669.72	160.76	<0.0001
AC	92.80	22.28	0.0033
AD	83.18	19.97	0.0042
BC	433.15	103.97	<0.0001
BD	277.64	66.64	0.0002
CD	143.92	34.55	0.0011
Residual	4.17	-	-
Lack of fit	0.044	0.0088	0.9289
Pure error	4.99	-	-

The "Lack of Fit P-value" determines how good the selected model is for prediction purposes. It is important to note that for a model to be considered a good fit, the "Lack of Fit P-value" must be > 0.5.

This is because a value of P < 0.5 means model "significantly lack fit"; in other words, the model is significantly not fit for prediction. That is, the model is significantly not fit for prediction. This is evident in Table 1, where the P-value for lack of fit is 0.9289. Component B, i.e. the mixture of surfactants Tween 80/Tween 85, was shown to have the biggest influence on the droplet size.

A response was investigated regarding outliers and all points were found to be in a normal distribution. Analysis of variance showed that the "Predicted R^2" of 0.9807 is in reasonable agreement with the "Adjusted R^2" of 0.9891. This model can be used to navigate the design space. The results show that more than 90 % of the droplet size response variation can be described by the mixture design model as a function of the main composition.

Based on the results obtained, the quadratic model proved to be a suitable model to analyse, predict and show the correlations between the variables. The interaction between variables was effective on droplet size and interaction AB (interaction between IPM and Tween 80/Tween 85) influence the response (droplet size) the most followed by BC (interaction between Tween 80/Tween 85 and glycerol) compared with other interactions.

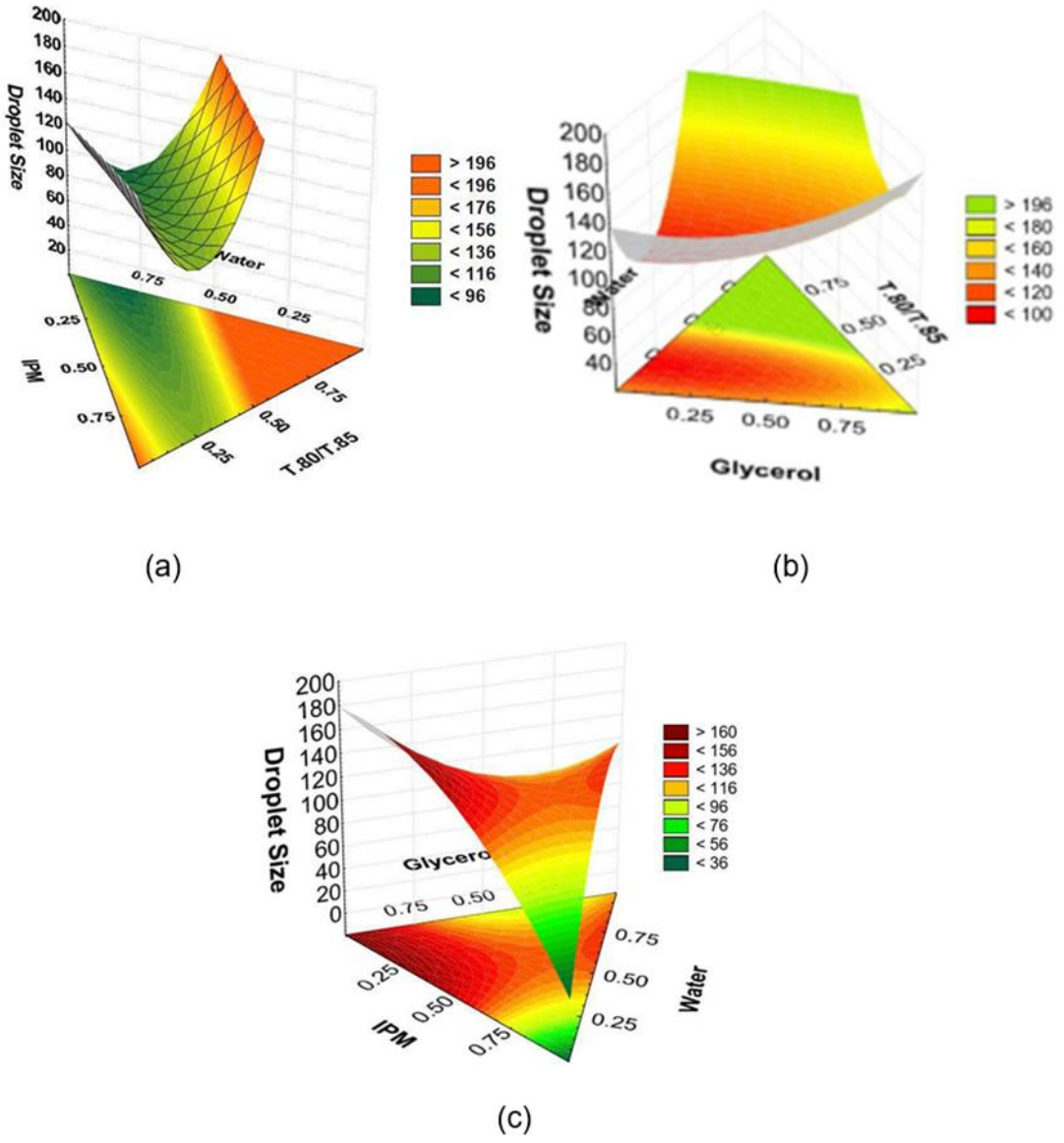


Figure 3. Ternary plots showing the interaction between (a) IPM, Tween 80/Tween 85 and water; (b) Tween 80/Tween 85, glycerol and water; (c) IPM, glycerol and water.

Response Surface Analysis

The interactions between four different components on the droplet size of nanoemulsions were analysed through the three-dimensional surface ternary plot. Figure 3(a) showed that as the concentration of oil (IPM) increases, droplet size also increases until it reaches the maximum shown by the shaded orange colour area. On the other hand, the smallest droplet size was obtained when the concentration of Tween 80/Tween 85 was increased. This is due to the sufficient amount of Tween 80/Tween 85 is available to emulsify the IPM with the aqueous phase as the amount of oil decreases. The plotted model depicted a linear increase in droplet size as the oil concentration increases.

The increased viscosity of the dispersed phase (oil phase) led to an increase in flow resistance, which may affect the droplet disruption process, causing large droplets to form during emulsification. When the concentration of Tween 80/Tween 85 used was reduced, the droplet size of the nanoemulsion was increased. This could be due to the fixed amount of the emulsifier leading to incomplete coverage of emulsifier molecules on the newly formed droplets. It can lead to an increase in the droplet size of an emulsion [24]. The pharmaceutical industry requires the formulation of nanoemulsions to have a very small droplet size as a small droplet size will result in low surface tension and reduces the interfacial tension of oil-in-water droplets [25].

Figures 3(b) and 3(c) showed that as the concentrations of glycerol and Tween 80/Tween 85 were increased, the droplet size decreased until minimum values in droplet size showed in the shaded orange and dark green color area. This observation has been previously reported, whereby the droplet size decreases due to more emulsifiers present to cover any new droplet surfaces formed during homogenization which leads to the reduction of the interfacial tension between oil and water [26, 27]. Increasing the amount of emulsifier decreases the interfacial tension which leads to the reduction of Laplace pressure and stress required for droplet deformation. These results showed that Tween 80/Tween 85 and glycerol contribute immensely to decreasing the droplet size of nanoemulsion.

Model Verification and the Optimum Formulation

Verification of the final model was done to counter-check the accuracy of the predicted response. Six random formulations with different percentages of components were prepared to validate the models. Actual values were compared with the predicted values to examine the adequacy of the final reduced model, as shown in Table 4. The residual standard error percentage (RSE, %) was calculated for each formulation result. This result confirmed the validity of the model, and the actual values were determined to be similar to the predicted values which indicated excellent fitness of the model generated. The optimum formulation was obtained based on the minimum droplet size of the nanoemulsion and the amount of the mixture of surfactants. Using this approach, a suitable composition of components was generated. Based on the MED analysis, a composition of 3.00 % IPM, 2.04 % Tween 80/Tween 85, 7.00 % glycerol, and 87.96 % water, was predicted that the nanoemulsion would have a droplet size of 99.58 nm as shown in Table 4. The desirability of the optimum formulation was 0.885. When the desirability value was between 0.8 and 1.0, the formulation quality was regarded to be acceptable and excellent. When this value was lower than 0.63, the formulation quality was regarded as poor [28].

Table 4. Validation set for the optimum formulation of phenytoin-loaded nanoemulsion.

	Components (% w/w)				Droplet size (nm)		RSE (%)
No	**IPM**	**Tween 80/Tween 85**	**Glycerol**	**Water**	**Actual value**	**Predicted value**	
Validation set							
1	5.00	2.25	5.50	87.25	118.3	116.82	1.27
2	4.50	1.80	5.50	88.20	137.0	133.56	2.58
3	4.50	2.10	5.50	87.90	114.0	118.31	3.64
4	4.50	2.20	5.50	87.80	112.1	114.24	1.87
5	4.50	2.40	5.50	87.60	109.0	107.62	1.28
6	4.50	2.25	5.10	88.15	110.7	112.94	1.98
Optimum formulation	3.00	2.04	7.00	87.96	99.80	99.58	0.885

Physicochemical Characterisation

Table 5 shows the physicochemical characteristic of the optimised formulation. The droplet size showed less than 100 nm, which would give better penetration through BBB [29]. The optimised formulation showed monodispersed droplet distributions with a PDI value of less than 0.250 and showed stable or no separation occurred for long-term stability, where the zeta potential value was -46.4 mV. For intravenous delivery, the pH of the nanoemulsion should be around 7.0 to 8.0 similar to the pH of the blood. However, due to solubility issues of phenytoin, the pH of parenteral phenytoin had to be adjusted to higher than 8.3 to avoid precipitation of the drug during administration. Hence, the pH of phenytoin-loaded nanoemulsion was adjusted to 10.23 ± 0.12 which was lower compared to marketed parenteral phenytoin sodium with a pH value of 12. The viscosity of nanoemulsion for parenteral administration is very important to avoid pain and irritation during the injection. Phenytoin-loaded nanoemulsion exhibited suitable viscosity reading for intravenous which was 1.37 cP, similar to the viscosity of water. The viscosity of the emulsion system would increase with the increasing concentration of the oil phase, therefore, the concentration of oil and surfactants must be less than 10 % to ensure the emulsion system does not become too viscous.

Table 5. Physicochemical properties of optimized nanoemulsion.

Paramater	The optimised formulation
Droplet size (nm)	98.69 ± 0.33
PDI	0.211 ± 0.03
Zeta potential (mV)	-46.4 ± 0.92
pH	10.2 ± 0.1
Viscosity (cP)	1.37 ± 0.15

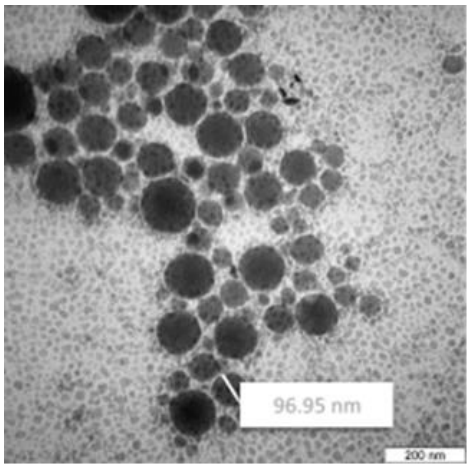


Figure 4. TEM image of phenytoin nanoemulsion.

Table 6. Entrapment efficiency study of the optimised nanoemulsion.

Period	**$W_{initial}$ (mg/30 mL)**	**$W_{obtained}$ (mg/30 mL)**	**Entrapment efficiency (%)**
1 month	36.0	0.3	99
2 months	32.0	0.4	99
3 months	28.0	0.8	97

Figure 4 showed the average droplet size diameter observed was found to be approximately around 90 nm to 100 nm, which was similar to the value obtained from zeta sizer analysis. TEM micrograph showed that the oil droplets were spherical with uniform size. Based on the HPLC chromatogram, it was shown that phenytoin was successfully encapsulated into the lipid carrier since no peak was observed at the same retention time, 9.8 mins (Result not shown here). Table 6 shows the results obtained for the entrapment efficiency test using Eq. 2. Almost 99 % of entrapment efficiency was detected for the phenytoin-loaded nanoemulsion. The results indicated that there was no major drug leakage; hence it was believed that phenytoin was encapsulated on the micelle.

Stability Studies of the Optimized Phenytoin-loaded Nanoemulsion

The analysis of the stability evaluation of phenytoin-loaded nanoemulsion within 3 months of storage at 25 °C was carried out concerning the droplet size, pH, and encapsulation efficiency. Storage of nanoemulsion at 4°C and 45°C were not studied as phenytoin was seen recrystallizing in the solution. It had been known that a lower temperature would shift the equilibrium facilitating the crystallization of phenytoin.

The droplet size of the formulation showed an increment after 90 days from 98.69 nm to 101.6 nm. PDI also showed an increment from 0.211 to 0.289. No phase separation was observed throughout 90 days for the formulations. Although the samples showed an increment, the droplet size value was still in the range of 100 nm. The good stability could be due to the good absorption of the mixed emulsifier on the droplet interface. Besides, NaCl produced during the neutralization process provided an extra layer of ionic protection preventing the coalescence of oil droplets.

Cytotoxicity Analysis

The cytotoxicity of phenytoin-loaded NE was investigated on Vero cells with Dilantin and blank nanoemulsion (blank NE) as standard. The IC_{50} values for each sample were determined. Figure 5 shows the cell viability at various concentrations for 72 h of exposure. A lower concentration indicates higher toxicity. For Dilantin and phenytoin-loaded NE, 50% inhibition occurred at a concentration of 210 µg/mL and 810 µg/mL, respectively. This indicated that phenytoin-loaded NE was four times less toxic than commercialized parenteral, Dilantin. As for blank NE, more than 60 % cell viability was recorded at a concentration of 1000 µg/mL, which indicated that the blank NE was non-toxic and safe to be used for parenteral purposes. The reason why Dilantin was more toxic than phenytoin-loaded NE was due to the high concentration of surfactant (30 % propylene glycol) and organic solvent (10% ethanol) in the solution maintained at pH 12. This study successfully proved that attaching phenytoin to nanoemulsions could significantly reduce its toxicity.

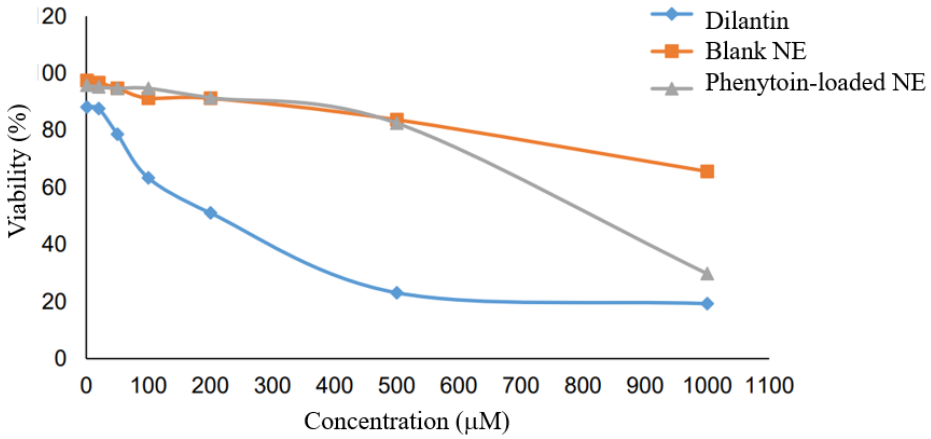


Figure 5. The number of cells viability at various concentrations for 72 h of exposure.

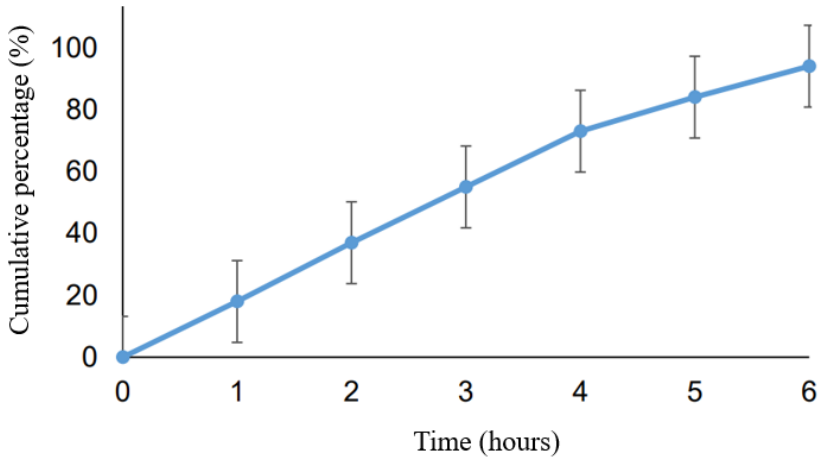


Figure 6. Drug release profile of phenytoin-loaded nanoemulsion.

In-vitro Drug Release Analysis

Figure 6 showed that the optimised formulation had a steady increase in drug release throughout 6 h of the experiment. Based on the release profile, it could be observed that 50 % of the drug was released within 3 h of dissolution. The highest cumulative percentage release of phenytoin was recorded at 97 % at 6 h. The sustained release of the drug could be due to its affinity to the oil phase as phenytoin was shown to be highly soluble in Tween 80 and Tween 85 based on the solubility test.

This could be attributed to its high partition coefficient (log P approximately 2.47). Additionally, the use of polymeric surfactants such as Tween 80 and Tween 85 could hinder the release of the drug due to its ability to form strong interfacial films surrounding the oil droplets. The presence of strong interfacial films also prevents drugs from being released drastically in a short period which could cause 'dose dumping' [30].

However, it is important to note that a high concentration of surfactants would further hinder the diffusion of drugs and a longer time was needed to achieve a peak plasma level, which is undesirable for parenteral delivery. The prompt and steady release of phenytoin-loaded nanoemulsion is useful when rapid serum concentrations are required, such as in the case of status epilepticus.

CONCLUSION

Encapsulated drugs on nanocarriers can be exploited to overcome the physiology of blood-brain barriers associated with drug delivery for complex diseases such as epilepsy. This study showed that encapsulating phenytoin in nanoemulsions was formulated through understanding the pH solubility profile of phenytoin. Compositions of the nanoemulsion were optimized through a D-optimal experimental design. The initial size of the droplets depended on the amount of oil, surfactant type, and amount of surfactants which are the major factors influencing nanoemulsion formation and stability. Phenytoin-loaded nanoemulsion with a droplet size of < 100 nm could be formed using 2.04% of a mix of non-ionic surfactant (Tween 80/Tween 85), 7.00% glycerol and 3% of IPM by controlling the processing parameter during formulation. In this study, we systematically examined some of the major factors influencing the encapsulation and stability of phenytoin in nanoemulsions. The nanoemulsion containing phenytoin remained physically stable during storage for three months at a temperature of 25 ± 2 °C. Based on the release profile, the highest cumulative percentage release of phenytoin was recorded at 97 % at 6 h. However, the efficiency of phenytoin-loaded nanoemulsion for the treatment of epilepsy needs to be further investigated.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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