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Development and Validation of Stability Indicating UPLC Assay method and Bioanalytical method by UPLC-MS/MS for Phenytoin & Development and Characterization of Phenytoin Nanoemulsion.

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ABSTRACT

A selective and sensitive analytical method that is reliable and reproducible was developed for the evaluation of phenytoin. Chromatographic separation was performed on Acquity UPLC using sunniest C18 column (2.1X50) mm, 2µm. An isocratic elution of mobile phase having composition of 0.5% formic acid buffer and ACN in 70:30 v/v ratio with flow rate as 0.2ml/min was used with UV detection at 215 nm. Calibration showed that the response of phenytoin was linear in the range of 80-120 µg/mL with $r^2 \geq 0.999$. The method was validated and was found to be linear, accurate, robust, specific, precise and rugged.

For rapid quantification of phenytoin in mice plasma a simple, accurate and precise Liquid Chromatographic method with mass detection was developed. Bio analysis was performed using Acquity BEH (50X2.1) mm, 1.7µm column. Mobile phase consisted of 100mM ammonium acetate: ACN: formic acid (50:950:2) and 100mM ammonium acetate: water: formic acid (50:950:2) as eluent at the rate of 0.8mL/min for 2.5 min. The effluence was ionized by Electron Spray Ionization (ESI), negative mode. Niflumic acid was used as the internal standard. The retention time for phenytoin and niflumic acid were found to be 1.30 and 1.71 min respectively. The calibration curve was linear with $r^2 \geq 0.99$ ranging from 100-5000 ng/mL with LLOQ to be 100 ng/mL. Interday and intraday precision was lower than 15% (CV), accuracy ranged from 85-115% and mean extraction recovery was found to be 99.72%. A lipid based formulation of phenytoin, a lipophilic drug was prepared by phase inversion composition (PIC) method. Solubility of phenytoin in different nanoemulsion components viz. oil, surfactant and co-surfactant was determined. Based on the solubility determination and emulsification properties Labrafac oil was selected, Solutol HS15 as the surfactant and Transcutol P as the co-surfactant. Surfactant and co-surfactant were mixed in different volume ratios (1:0, 1:1, 1:2, 2:1, 3:1 and 4:1). Phase diagrams were developed using aqueous titration method as per the titration charts. Ternary phase diagrams were used to evaluate the nanoemulsion existence area. Placebos and then selected formulations NE-B1 and NE-B4 were subjected to thermodynamic stability tests. Formulations were characterized for viscosity, %transmittance, refractive index, droplet size and zeta potential. Droplet size of the optimized formulations was found NE-B1 was 21nm and NE-B4 was 20.44nm respectively and zeta potential was found to be -33.7 and 33.94 mV respectively. Pharmacokinetic studies showed 14.4 folds and 2.7 fold increase in the bioavailability of phenytoin from NE-B1 and NE-B4 respectively.

Introduction

Ultra Performance Liquid Chromatography (UPLC) can be regarded as new invention for liquid chromatography. UPLC has brought dramatic improvements in sensitivity, resolution and speed of analysis. It has instrumentation that operates at high pressure than is used in HPLC and in this system uses fine particles (less than 2.5µm) and mobile phases at high linear velocities decreases the length of column, reduces solvent consumption and saves time.

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UPLC is preferred over HPLC because it offers high speed, less time consumption and better productivity. Due to very narrow and sharp peaks more number of peaks may appear in less time which may facilitate in analysis of complex mixture and it may give more information regarding samples to be analyzed. Very low dead volume in the system (due to smaller column, narrower internal diameter etc.) makes this an excellent LC system with significant advantage over conventional HPLC system. UPLC also lifted the pressure limit up to 15,000psi from a typical 4000psi of HPLC.UPLC removed the barrier of traditional chromatography packing material by development of new, highly efficient, mechanically strong, 1.7 mm bridge hybrid particles that are stable over a broad pH operating range.

Stress testing is a part of developmental strategy under the ICH requirements and is carried out under more severe conditions than accelerated conditions. These studies serve to give information on drug's inherent stability and help in the validation of analytical methods to be used in stability studies. It is suggested that stress testing should include the effect of temperature, light, oxidizing agents as well as susceptibility across a wide range of pH values. It is also recommended that analysis of stability sample should de done through the use of a validated stability testing methods. (H.S. Joshi et al., 2011)

Analytical methods employed for the quantitative determination of drugs and their metabolites in biological samples are the key determinants in generating reproducible and reliable data which in turn are used in the evaluation and interpretation of bioavailability, bioequivalence, and pharmacokinetic findings. It is essential to employ well-characterized and fully validated analytical methods to yield reliable results which can be satisfactorily interpreted. There have been tremendous advancements in the field of mass spectrometry with the development of new interfaces, ionization and detection techniques. These advancements resulted in the rapid emergence and wide- spread commercial use of hyphenated mass spectrometry (LC-MS-MS) based assays, which have largely replaced conventional HPLC, GC and GC-MS assays (Shah et al., 2000).

Phenytoin is an antiepileptic drug that belongs to the class of anticonvulsants. Phenytoin is chemically 5,5-Diphenylhydatoin having chemical formula $C_{15}H_{12}N_2O_2$ and molecular weight 252.268. It controls generalized tonic-clonic (grand mal) and complex partial (psychomotor, temporal lobe) seizures and prevents and treats seizures occurring during or following neurosurgery.

Phenytoin is a BCS class II drug having low solubility and high permeability. Nanoemulsions (NEs) improve the bioavailability by increasing the solubility of hydrophobic drugs and are now widely used for the administration of BCS class II and IV drugs (Dahan and Hoffman, 2007). Nanoemulsion offers a major advantage of being dose adjustable. Since nano emulsions are liquid dosage forms, it offers the superiority of dose adjustment. The dose can be easily adjusted for pediatric population based on body weight or surface area. Oral NEs use safe edible material [e.g., food grade oils and "Generally Regarded as Safe" (GRAS) grade excipients] for fabrication of the delivery systems (Foguet et al., 2009; Tiwari et al., 2006).

MATERIAL

Phenytoin (Sigma Aldrich, USA), Eptoin Tablet formulation (Abott India Ltd, Acetonitrile for UPLC (Spectrochem Pvt. Ltd., Mumbai), Hydrogen per oxide (Spectrochem Pvt. Ltd., Mumbai), Sodium hydroxide pellet (Thomas Baker (chemicals) Pvt. Ltd. Mumbai, India), Hydrochloric acid (RFCL Limited, Delhi, India), Formic acid (Sigma Aldrich, USA), Niflumic acid (Sigma Aldrich, USA), Labrafac Lipophile WL 1349 (Gattefosse, Saint Priest, Cedex, France), Captex GTO (Abitec Corporation, Janesville), Polyethylene Glycol 400 ((Thomas Baker (chemicals) Pvt. Ltd. Mumbai, India), Dialysis bag (MWCO 1200) (Hanson Research SR8 Plus, California, USA)

EQUIPMENTS

UPLC (Waters Milliford, USA), Analytical balance (Satorious, Goenttingen, Germany), Hot Air Oven (Shel Lab, Wilmington, USA), Digital pH Meter (Sartorious, Goenttingen, Germany), Milli Q Water System (Millipore, Massachusetts, Germany), Thermo lab Stability Chamber (Effem Technologies, Mumbai, India), Biological Shaker (Nirmal International, Delhi, India), Centrifuge Apparatus (Remi Equipment, Delhi, India), Deep Freezer(-80^{o}C) (Thermo Electron Corp), Micropipettes (Eppendorf micropipette, Chennai, India), Particle Size Analyzer Zeta sizer (Malvern Instrument, Worcestershire, U.K.), Refractometer (Abbes, India), Transmission Electron Microscopy (FEI, Eindhoven, Netherlands), Sonicator (Bio Technics, Mumbai, India), Viscometer (Brookfield Engineering Laboratories, MA, USA), Mass spectroscopy (API 4000) (AB Applied Biosystems)

A. SIAM

CHROMATOGRAPHIC CONDITIONS

Chromatographic analysis for SIAM was performed on Acquity UPLC^{TM} system (Waters, Milliford, USA) consisting of binary solvent manager, sample manager and PDA detector. System control, data collection and data processing were done using Waters Empower^{TM} chromatography data software. Optimizations of chromatographic conditions were performed to obtain the good peak shape and peak parameters (tailing factor, theoretical plates). The analytical column used was Sunniest C18-HT, 2µm 2.1mmi.d.x 50mm and separation was achieved using isocratic elution. 0.5% formic acid and acetonitrile was used as the mobile phase (70:30v/v) at a flow rate of 0.2 mL/min, injection volume as 2µL , column temperature 40^{o}C, detection wavelength 215nm and run time of 5 min.

STANDARD PREPARATION

100 mg of standard substance was weighed accurately and transferred to 100 mL volumetric flask. About 10 mL of diluent was added to the flask and sonicated for 2 min. It was then diluted to the mark with diluent and further sonicated for 5 min. The resulting solution was filtered using 0.2 µm filter. 10 mL of this filtrate was transferred to 100 mL volumetric and diluted to volume with diluents and sonicated to get a solution of strength 100 µg/mL.

SAMPLE PREPARATION

One tablet (Eptoin, 100 mg) was taken in a 100 mL volumetric flask. About 10 mL of diluent was added to the flask and sonicated for 2 min. It was then diluted to the mark with diluent and further sonicated for 5 min. The resulting solution was filtered using 0.2 µm filter. 10 mL of this filtrate was transferred to 100 mL volumetric and diluted to volume with diluents and sonicated to get a solution of strength 100 µg/mL.

METHOD VALIDATION

System Suitability- To verify the performance of the system, system suitability was measured. It was determined on six injections of standard preparation. All the important parameters were measured.

Specificity-The ICH guidelines entitled stability testing of new drug substance and products that requires stress testing was carried out to elucidate the inherent stability characteristics of the active substance.

Forced degradation studies were performed on drug substance (except photo-stability on drug product) to establish inherent stability characteristic in order to demonstrate selectivity and stability indicating capability of method. The standard substance was exposed to following stress conditions:

(a) Acidic stress studies were performed by exposing the standard solution to 0.1-5 N HCl and the solutions were refluxed for 8 h every time.

(b) Alkaline stress studies were done with increasing strength of NaOH i.e. from 0.1-8N NaOH and refluxed for 8 h.

(c) Strong oxidizing agent H_2O_2 of strength varying from 3-30% was added to the standard solution and was kept at 80^{o}C.

(d) Neutral stress studies were carried out with H_2O and keeping the solution at 60°C for 4 h.

(e) Thermal degradation study was done by keeping the drug substance at 80°C for one day in an oven

(f) Photolytic degradation study was carried out on tablet formulation of phenytoin at 254nm for 1day.

Blank solutions were subjected to stress in same manner and all the samples subjected to analysis in accordance with the developed method.

Linearity- Under the proposed experimental conditions, peak area of standard substance was plotted against concentration of phenytoin. Linearity was demonstrated from 80-120% using minimum five calibration levels (80, 90, 100, 110 and 120µg/mL). Linearity was described by an equation and correlation coefficient was determined.

Precision- Repeatability and intermediate precision was carried out through six injections of samples of commercial brand (Eptoin, Abbot India) of tablets and analyzing by proposed method. Intermediate precision was performed by carrying out the analysis on different column and different day.

Accuracy-To find the accuracy of the method, the recovery experiment was carried out using the standard addition method. For the previously analyzed tablet sample, a known amount of standard was added at 50%, 100% and 150% level and it was analyzed in triplicate. The % recovery of drug at each level and each replicate was determined. The mean of percentage recoveries and the RSD was calculated.

Limit of Detection (LOD) and Limit of Quantification (LOQ) - LOD and LOQ were determined using the following equations:

$LOD = 3.3x\sigma/S$ and

$LOQ = 10x\sigma/S$,

where, σ = standard deviation of the calibration curve

S = slope of the calibration curve

Robustness- Capacity of a method to remain unaffected by small but deliberate changes in the chromatographic conditions was studied by testing influence of small change in pH of buffer (±0.2 units), column temperature (±5%), organic content of mobile phase (±2%) and flow rate (±5%).

Ruggedness - The ruggedness of the method was determined by carrying out the analysis using two different analysts on two different days and the respective mean areas were noted and RSD was calculated.

Accelerated stability study

Stability of sample preparation- Stability of sample solution was determined by storage of the sample solution at ambient temperature (32^oC) for 24 hrs. Sample solution was reanalyzed after 24 hrs and was determined and compared against fresh sample.

Stability of marketed formulation- Stability of marketed formulation was determined by storage of sample solution at $40±2^oC/75±5\%$ RH. Sampling was done for seven days to investigate any degradation products.

B. BIOANALYTICAL VALIDATION

CHROMATOGRAPHIC CONDITIONS

For Bioanalytical validation, chromatographic analysis was performed on API-5500 AB Sciex (Applied Biosystems) using Acquity UPLC system as the autosampler. Analyst 1.5.1 software was used for the collection and processing of the data. The analytical column used was Acquity BEH C18, (50 x 2.1) mm, 1.7µ and separation was achieved using gradient elution. 100mM Ammonium Acetate: ACN: Formic Acid (50:950:2) and 100mM Ammonium Acetate: Water: Formic Acid (50:950:2) at a flow rate of 0.8 mL/min, injection volume as 10µL , column temperature 50^oC and run time of 2.5 min.

STANDARD PREPARATION

Preparation of the stock solution

1mg/mL phenytoin solution was prepared by weighing accurately about 2 mg phenytoin standard and dissolved in measured volume of acetonitrile.

Internal standard solution Preparation

Niflumic acid of concentration 0.01µM was used as the internal standard solution.

Development of phenytoin mass parameters

Stock solution of phenytoin diluted using acetonitrile to obtain a concentration of about 20ng/mL. The diluted solution was directly infused into the LC-MS/MS using infusion pump and mass parameters were optimized. Similarly niflumic acid parameters were also optimized.

Preparation of Linearity solutions

Series of working standard solutions with concentration of 5000, 4000, 4500, 3000, 2000, 1000, 500, 300, 400, 200, 100, 50ng/mL was prepared by appropriate dilution of stock solution using acetonitrile.

Preparation of Quality control solutions

Low, medium and high quality control solutions (4000, 1000 and 400 ng/mL respectively) were also prepared by diluting stock solution using acetonitrile.

Standard Linearity Curve Preparation

To 20 µL of blank plasma 25 µL Milli-Q water was added. It was centrifuged gently to bring down all the contents to bottom of the tube. Then 20 µL of the linearity solution was added. To this 200 µL internal standard solutions was added. It was mixed on 96 well shaker for about 1minute. Similarly Quality control samples were also prepared using quality control solutions.

SAMPLE PREPARATION

To 20 µL of plasma sample 25 µL Milli-Q water was added. It was centrifuged gently to bring down all the contents to bottom of the tube. To this 20 µL acetonitrile and 200 µL internal standard solution was added. It was mixed on 96 well shaker for about 1minute.

Blank Preparation

Blank plasma was taken instead of plasma sample and preceded as described in sample preparation.

Filtration

All the preparations were filtered using Captiva 96 well polypropylene filter plate and directly injected.

VALIDATION

4.8.1. Selectivity: The selectivity of the assay methodology was established using independent sources of the same matrix.

4.8.2. Preparation of calibration curve: The linearity of the method was evaluated by a calibration curve in the range of 5000 – 100 ng/ mL of phenytoin, including lower limit of Quantitation (LLOQ). The calibration curve was achieved by plotting the peak area ratios of phenytoin and internal standard versus the concentration of phenytoin by least-squares linear regression analysis. The calibration curve requires a correlation coefficient (r^2) >0.99. The acceptance criteria for each back-calculated standard concentration should be within 15% of the nominal concentration, except it should not exceed 20% for the LLOQ. Each validation run consisted of a double blank, system suitability sample, a zero standard, calibration curve consisting of non-zero samples covering the total range (5000-100 ng/mL) and QC samples at three concentrations. Such validation runs were generated on three consecutive days.

4.8.3. Precision and Accuracy

The precision of the developed method was determined by analysis of three different quality control samples containing 400, 1000 and 4000ng/mL (n=6). To validate the method for phenytoin, intraday accuracy and precision were evaluated by analysis of three different concentration levels (LQC, MQC, HQC) on same day and inter day accuracy and precision were evaluated by analysis at three different concentration levels (LQC, MQC, HQC) other day.

4.8.4. Recovery

Recovery of phenytoin was evaluated by comparing the mean peak areas of three extracted low, medium and high quality (4000, 1000 and 400 ng/mL) control samples to mean peak areas of three neat reference solutions (un-extracted). Recovery of the analyte need

not be 100%, but the extent of recovery for analyte (phenytoin) should be consistent and reproducible.

4.9. Stability

Stock solution was stored at 4-8 ºC. Drug stability was checked for two different concentrations HQC and LQC. Freeze thaw cycles were performed for three cycles to check the influence of temperature on the stability of drug and it was analyzed after every 24hr. Samples were stored at 4-8 ºC and thawed at 37ºC in an incubator.

C. DEVELOPMENT AND CHARACTERIZATION OF PHENYTOIN NANOEMULSION

Formulation of nanoemulsion by Phase Inversion Composition (PIC) method

Screening of oil

Materials used for oral application should be pharmaceutically acceptable and should fall under GRAS (Generally Regarded As Safe) category. Selection of an appropriate oily phase is very important as it influences the selection of other ingredients of nanoemulsions, mainly in case of O/W nanoemulsions. Usually, the oil which has maximum solubilizing potential for selected drug candidate is selected as an oily phase for the formulation of nanoemulsions. This helps to achieve maximum drug loading in the nanoemulsion. Solubility of phenytoin was ascertained in different Medium Chain Triglyceride (MCT) oils like Vitamin E, Captex GTO, Sunflower oil, Coconut oil Labrafac and Plurol Oleique using shake flask method.

Selection of surfactant and co-surfactant

The surfactant should favour micro emulsification of the oily phase and should also possess good solubilizing potential for the hydrophobic drug compounds. Most of the times, surfactant alone cannot lower the oil-water interfacial tension sufficiently to yield a nanoemulsion which necessitates the addition of cosurfactant to bring about the surface tension close to zero. Surfactant and co-surfactant are selected on the basis of miscibility with the selected oil. Various. Tween 80, Tween 20, Solutol HS15, PEG 200, PEG 400, Transcutol HP, Ethanol and Propylene Glycol were screened.

Drug-excipient interaction studies

Phenytoin-excipients interaction studies were performed with the selected oil and excipients showing maximum solubility. Phenytoin and excipients were hermatically sealed and stored at regulated temperature and humidity (30±2 ^{o}C/ 65±5 %RH). After 30 days, physical observation was done and the content of phenytoin was analyzed by UPLC.

Construction of Pseudo-Ternary phase diagram

On the basis of the results obtained from the solubility studies, Labrafac was used as the oil phase, Solutol HS15 as the surfactant and Transcutol HP as co-surfactant for the formulation of nanoemulsion. Surfactant and co-surfactant were mixed (S_{mix}) in different volume ratios (1:0, 1:1, 1:2, 2:1, 3:1 and 4:1). Oil and specific S_{mix} ratio were mixed well in different ratios ranging from 1:9 to 9:1. Phase diagrams were developed using aqueous titration method as per the titration chart.

Selection of S_{mix} ratio

Solutol HS15 was used as the surfactant and Transcutol HP was used as the co-surfactant. Surfactant and co-surfactant were mixed in different ratios (1:0, 1:1, 1:2, 2:1, 3:1 and 4:1). Double distilled water was used as the aqueous phase. The amount of aqueous phase was varied. After each addition of the aqueous phase, physical state of the mixture was marked whether it is transparent or opaque. Oil and S_{mix} ratio was mixed in different volume ratios ranging from 1:9 to 1:0.1 to obtain 16 different combinations like 1:9, 1:8, 1:7, 1:6, 1:5, 1:4, 1:3.5, 1:3, 1:2.3, 1:2, 1:1.5, 1:1, 1:0.7, 1:0.43, 1:0.25 and 1:0.1.

Pseudo-Ternary phase diagrams were developed using the aqueous titration method. The nanoemulsion regions were marked on Pseudo-Ternary phase diagram with one axis representing the aqueous phase, the second one representing the oil (Labrafac) phase and the third axis representing the S_{mix} at a fixed ratio. The combination which produced the largest area for the nanoemulsion was determined.

Physical stability studies

Placebos and selected formulations were subjected to different thermodynamic stability tests to assess their physical stability.

1. Heating–cooling cycle: cycle between 25±2°C and 45±2°C with storage at each temperature for not less than 24 h were conducted, and the formulations were examined for stability at these temperatures.

2. Centrifugation test: Formulations were centrifuged at 5000 rpm for 20 min, and examined for phase separation.

3. Freeze–thaw cycle: The formulations were subjected to freeze– thaw cycles between −21±2°C and +25±2°C and observed for any phase separation

4. Dispersibility: Formulations were dispersed in distilled water and was checked.

Characterization of nanoemulsion

1. Refractive Index

The refractive index of the system was measured by an Abbe refractometer by placing one drop of the formulation on the slide in triplicate at 25±2°C.

2. Percentage Transmittance

Percentage Transmittance of the prepared nanoemulsion formulation was determined spectrophotometrically using UV-VIS spectrophotometer (Shimadzu, Koyoto , Japan). 1 ml of the formulation was diluted 10 times and analyzed.

3. Droplet size analysis

Zetasizer measures particle and molecule size from below a nanometer to several microns using dynamic light scattering. Droplet size of the nanoemulsion was determined by using Malvern Zetasizer (Malvern Instruments Ltd, United Kingdom). The formulation was diluted 100 times with distilled water and sonicated for 10 min before it was analyzed. Light scattering was monitored at 25°C at a scattering angle of 90°.

4. Transmission Electron Microscopy (TEM)

TEM is a microscopy technique where by an image is formed from the beam of electron transmitted through the sample, magnified and focused by objective lens and appears on the imaging screen. The morphology of the oil droplet or any precipitation of the drug upon addition of the aqueous phase in the nanoemulsion was observed with this technique. The nanoemulsion was diluted 100 times and a drop was applied to 300-mesh copper grid. The grid was left for one minute. The grid was inverted and a drop of phototungstic acid was applied to the grid for 10 s. Excess of phototungstic acid was removed using a fiter paper and it was analyzed

5. ***Viscosity***

The viscosity of the formulation (0.5ml) was determined at 25±2 °C using a Brookfield DV III ultra cone and plate viscometer (Brookfield Engineering Laboratories, MA, USA). The software used for the calculations was Rheocalc V2.6. The viscosity of the nanoemulsions with Newtonian flow properties was calculated.

6. Zeta Potential

Zeta potential were measured by photon correlation spectroscopy (PCI) using a Malvern particle sizer (Malvern Instruments Ltd, United Kingdom) with inbuilt software based on the electrophoretic mobility of globules and the Helmholtz-Smoluchowski equation

Zeta potential (Zp) = $6\pi\upsilon\eta/\varepsilon\chi$

Where, Zp is in volts, υ = migration velocity cm/sec, η = viscosity of the medium in poise, ε = dielectric constant of the external medium, and χ = potential gradient in volts. The formulation was diluted 100 times with double distilled water and was than analyzed.

***In*- Vitro studies**

Based on the drug content determination, 1 mL of nanoemulsion containing 5mg phenytoin was filled in pre-treated dialysis membrane. The dissolution was carried out using dissolution test apparatus USP Type II, at 37±5° C, 50 rpm paddle speed. Dissolution was performed in 900 mL of 6.8 pH Phosphate buffer. The samples were withdrawn at predetermined time intervals and were

analyzed for drug concentration by UPLC after filtration through 0.22 µ filter

***In*- Vivo studies**

Pharmacokinetics Application: Approval was obtained from Institutional Animal Ethics Committee, Daiichi Sankyo Pharma India Pvt. Ltd (Protocol no-DS2014/136P). The study was conducted in accordance with the Ethical Guidelines. Pharmacokinetics parameters of the optimized formulations NE B1 and NE B4 were evaluated and were compared with phenytoin suspension. Swiss mice (female) weighing 20-23 g were taken and randomly divided into three groups: NE (B1), NE (B4), and Suspension, each containing two mice. The animals were kept under standard laboratory conditions with temperature of 25±1º C and relative humidity of 55±5 %RH. The animals were housed in polypropylene cages, two per cage, with free access to a standard laboratory diet (Lipton feed) and water *ad libitum*. The dose of the mice was calculated based on the weight of the mice. Before the oral administration mice were fasted overnight. Animals were anesthetized with ether before to collect the samples. After the animals were dosed, blood samples were taken at an interval of 0.25, 0.5, 1, 2, 4, 8, 24 h in micro centrifuge tubes already containing 6 µL of anticoagulant (2% w/v sodium citrate). The samples were centrifuged for 5 min at 10,000 rpm and separated plasma layer were collected in duplicate and stored in deep freezer set at -80ºC. All the formulations were analyzed by already developed and validated bioanalytical method by using LC-MS-MS (Part A) and compared by using various PK parameters.

Data Analysis

PK parameters such as the peak plasma concentration (C_{max}), time to reach this peak (T_{max}) and AUC_{inf} were calculated using 'WinNonlin' software.

RESULTS

A. SIAM

Development and optimization of UPLC method

The main aim was to develop selective and sensitive analytical method for the quantitative evaluation of phenytoin. Method all the procedures that demonstrate that a particular procedure is reliable and reproducible. It should be able to determine assay of drug and should be accurate, precise, robust, free of interference from degradation product thus stability indicating also and could be applied for routine use in QC laboratory.

The increase in the column length resulted in the increase of the retention time. Therefore a shorter column having length of 50x2.5mm was selected to have shortest possible run time. Lower particle size (2.5µm) was selected to have good resolution between drug and dgradation product peaks.

0.5% formic acid buffer of pH-3.0 was found appropriate for elution of compound of interest. acetonitrile was used as the organic modifier because of its lower viscosity than methanol.

Minute change in the percentage of includes acetonitrile produced poor peak with high base line noise and a great difference in the retention time time. Therefore an isocratic run was chosen with regards to the peak shape, peak tailing and analysis time.

System suitability

A system suitability test of the chromatographic system was performed before each validation run. Six injections of standard preparation were injected tailing, theoretical plate and % RSD of peak area were determined for same. For all system suitability injections theoretical plate was greater than 1000 and % RSD of peak area was less then 2.0 found. Figure 1 and Figure 2 represents the chromatograms for the standard and test.

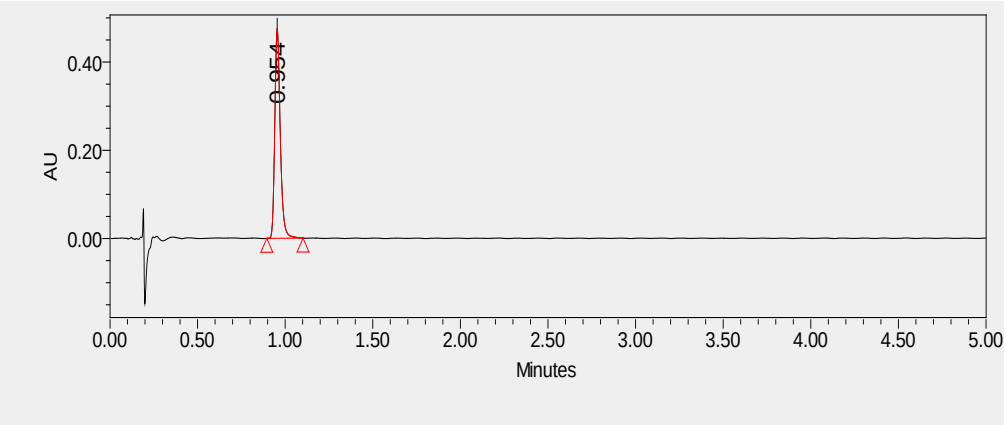

Figure 1: Chromatogram for phenytoin standard.

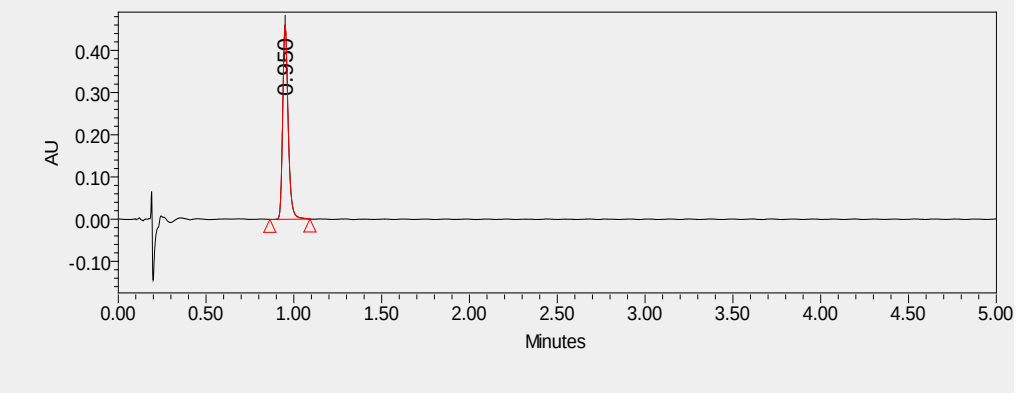

Figure 2: Chromatogram of phenytoin test (tablet solution)

Specificity

The specificity of the method was determined by checking the interference of analyte with degradarion product and the proposed method was eluted by checking the peak purity of phenytoin during the force degradation study. The peak purity of the phenytoin was found satisfactory under different stress condition. No degradation product peak was observed under various conditions. Major degradation was found in oxidative condition which was 1.9% (Fig. 3). In alkali degradation, it was found that around 1.4 % of the drug degraded (Fig. 4) and in neutral and oxidative conditions around 1.7 % of the drug degraded. Phenytoin was found to be stable under the thermal and photolytic conditions. Overall degradation less than 2% and absence of product peak in all conditions showed phenytoin as stable under various stress conditions.

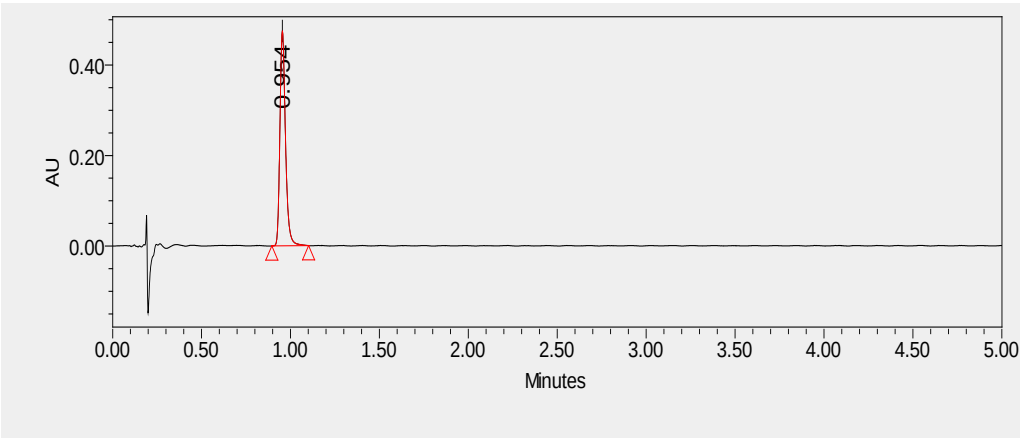

Figure 3: Chromatogram for stress study under acidic condition(5N HCl, 8 h reflux)

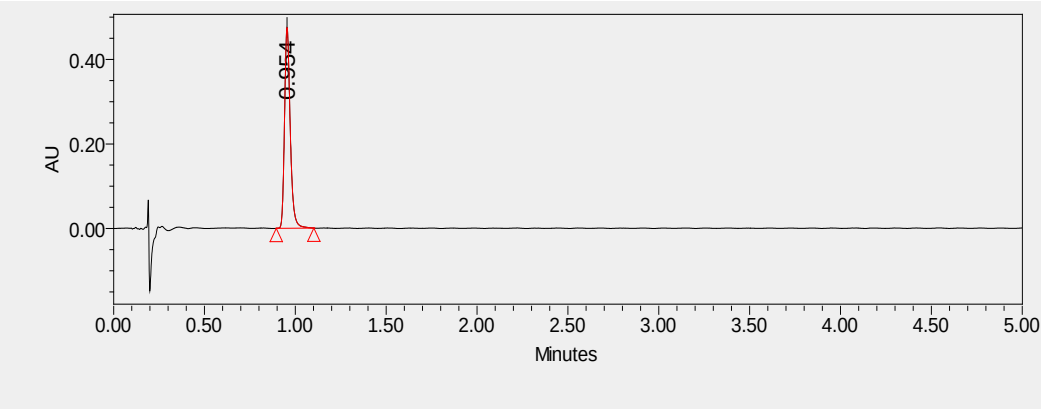

Figure 4: Chromatogram for stress study under alkaline condition (8N NaOH, 8 h reflux)

Linearity

Five points calibration curve were obtained in a concentration range from 80-120 µg/ml for phenytoin. The response of the drug was found to be linear in the investigation concentration range and the linear regression equation was y= 12247x + 29638 with correlation coefficient 0.9999.

Precision

The result of repeatability and intermediate precision study are shown in Table I. The developed method was found to be precise as the %RSD values for the repeatability and intermediate precision studies were 0.7 % and 0.8% respectively, which confirm that method was precise.

TABLE 1: Repeatability and intermediate precision.

SAMPLE NO.	% Assay Repeatability	% Assay Intermediate precision
1	98.863	100.143
2	100.234	98.547
3	100.008	98.907
4	100.104	99.980
5	98.877	98.289
6	100.535	99.689
MEAN	99.770	99.259
% RSD	0.7	0.8

ACCURACY

The UPLC area responses for accuracy determination are depicted in Table II. The results showed the best recoveries i.e 99.90, 99.72 and 99.93% with 50, 100 and 150% level of the spiked drug with each added concentration. RSD was found to be less than 2% indicating that the method was accurate.

TABLE 2: Evaluation data of accuracy study.

Level	Concentration (µg/mL)	Mean %Recovery (n=3)	%RSD
50	150	99.9	0.03
100	200	99.72	0.42
150	250	99.93	0.2

ROBUSTNESS

Capacity of a method to remain unaffected by small but deliberate changes in the chromatographic conditions was studied by testing influence of small change in pH of buffer (±0.2 units), column temperature (±5%), organic content of mobile phase (±2%) and flow rate (±5%). The method was found to be robust w.r.t. changes in above conditions.

RUGGEDNESS

TABLE 3: Evaluation data of ruggedness study.

S.No.	Analyst 1 Area(µV*sec)	Analyst 2 Area(µV*sec)
1	1259301	1249357
2	1269321	1259123
3	1249365	1245931
4	1272154	1239637
5	1269391	1243988
6	1290367	1236752
MEAN	1268317	1245798
%RSD	1.08	0.63

RSD was found to be less than 2% when the analysis was carried out by two different analysts on two different days. Hence the method was found to be rugged.

Limit of Detection (L.O.D) Limit of Quantification (L.O.Q):

Limit of Detection was found to be 1.64 µg/mL

and Limit of Quantification was found to be 4.97 µg/mL.

Stability of sample preparation

Table 4: Assay results of phenytoin after storage of sample solution for 24 h.

Hours	Area (µV*sec)	%Assay
0	1266720	99.9
24	1256648	99.1
		Mean=99.5

No appreciable change (table 4) was found when sample solution was kept for 24 h at ambient temperature(32^{o}C).

Stability of marketed formulation

Table 5: Assay results of phenytoin after subjecting sample solution to accelerated conditions.

Days	Area (µV*sec)	%Assay
1	1267210	100
5	1264379	99.8
10	1264051	99.8
15	1259070	99.4
20	1263637	99.7
25	1261963	99.6
30	1262173	99.6
Mean	1263211.857	99.7

When the tablet sample solution was subjected to accelerated conditions for one month, no change in the drug content was observed (table 4). Hence it was found to be stable.

B. BIOANAKYTICAL METHOD

Selectivity and optimization of chromatographic conditions

Plasma matrices were obtained from six different sources and assayed to evaluate the selectivity of the method and the detection of interference. Phenytoin and niflumic acid (internal standard) were well separated from the co-extracted material under the described chromatographic conditions at retention times of 1.30 and 1.71 min respectively. No endogenous peak from plasma was found to interfere with the elution of either the drug or the internal standard. The LLOQ which could be measured with acceptable accuracy and precision for the analyte 100 ng/mL was established (Figure 6).

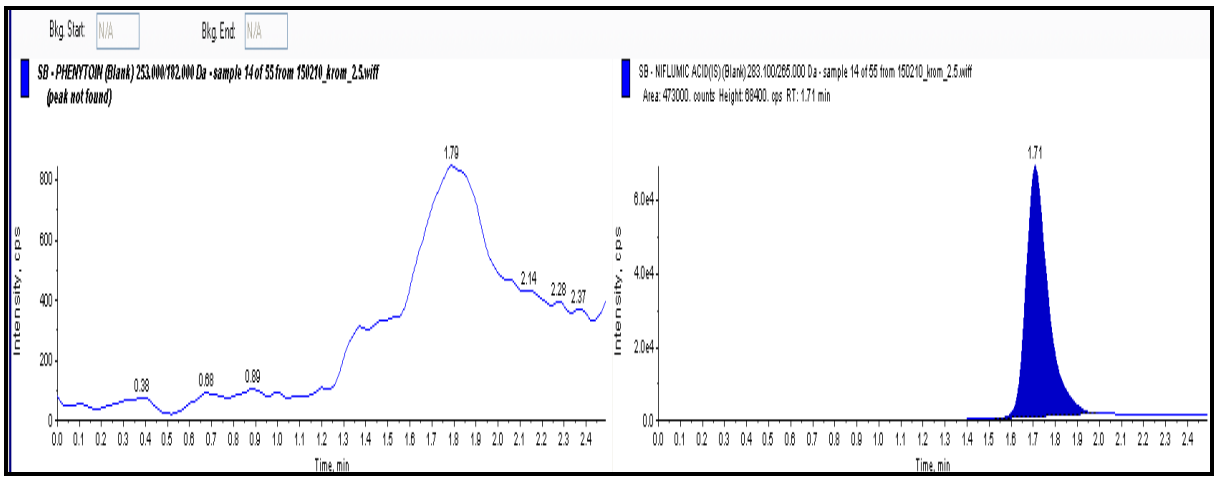

Fig 5: Representative chromatogram of blank A. Phenytoin B. Niflumic acid

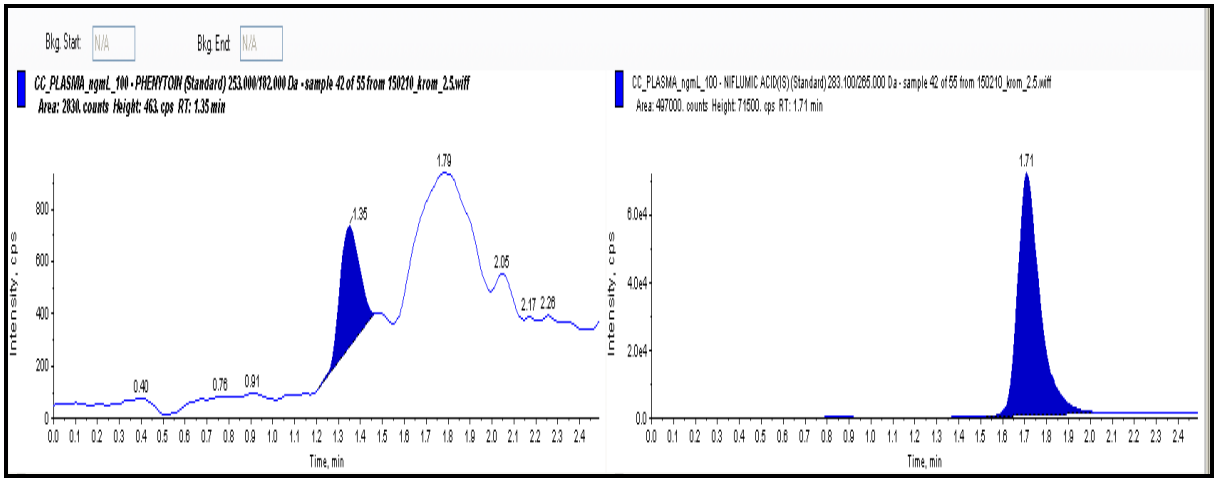

Fig 6: Representative chromatogram of LLOQ

LINEARITY

Linearity was studied over concentration range of 5000 - 100 ng/mL for phenytoin with correlation coefficient r^2>0.99. The calculation was done by linear weighed regression ($1/X^2$). The results obtained for linearity are listed in Table 6.

Table 6: Data of correlation coefficient (r^2)

LINEARITY			
DAY	**SLOPE**	**INTERCEPT**	**REGRESSION**
1	1.0125	22.0360	0.9891
2	0.9737	32.2670	0.9911
3	0.9674	20.0560	0.9969
MEAN	0.9845	24.7863	0.9924

ACCURACY AND PRECISION

The method shows inter day precision and accuracy for LQC, MQC and HQC levels (400, 1000 and 4000 ng/mL) are 4.51, 3.17, 3.95% and 105.083, 104.883 and 105.916% as shown in Table 7. And intraday precision and accuracy for LQC, MQC and HQC levels (400, 1000 and 4000 ng/mL) are 12.327, 6.308, 9.783% and 107.25, 111.333 and 109.916%. (Table 8). Percentage coefficient of variation was found to be less than 15% at each level of quality control. Therefore the method was found to be precise and accurate.

Table 7: Data of inter day precision.

INTER- DAY PRECISON				
QC LEVEL	**NOMINAL CONC. (ng/mL)**	**MEAN OBSERVED CONC. (ng/mL)**	**%ASSAY**	**%CV**
LQC	400	420.333	105.083	4.51
MQC	1000	1048.333	104.833	3.17
HQC	4000	4236.666	105.916	3.95

Table 8: Data of intra day precision.

INTRA- DAY PRECISON				
QC level	**NOMINAL CONC. (ng/mL)**	**MEAN OBSERVED CONC. (ng/mL)**	**%ASSAY**	**%CV**
LQC	400	429	107.25	12.327
MQC	1000	1113.333	111.333	6.308
HQC	4000	4396.667	109.916	9.783

RECOVERY

The extraction recovery of the developed method was determined at three different quality control levels LQC, MQC and HQC i.e. 400, 1000 and 4000 ng/mL respectively. It was found to be 91.116, 94.874%and 113.17% for LQC, MQC and HQC respectively. The percentage recovery of phenytoin and niflumic acid (internal standard) were found to be above 80%. Results are displayed in the Table 9.

Table 9: Data of extraction recovery

EXTRACTION RECOVERY			
QC LEVEL	**NOMINAL CONC. (ng/mL)**	**MEAN EXTRACTED RECOVERY %**	**%CV**
LQC	400	91.116	4.097
MQC	1000	94.874	5.054
HQC	4000	113.17	5.42

STABILITY

Stock solutions of phenytoin and internal standard were separately prepared. The solutions were stable for at least 1 month when stored at 4°C. The stability experiments were aimed at testing all possible conditions that the samples might experience after collecting and prior the analysis. Stability results were summarized in Table 10. The results of three freeze– thaw cycles indicated that phenytoin was stable in rat plasma under these conditions. Analyte spiked QC (HQC and LQC) samples were stable for at least 30 days if stored in the freezer at 4−8°C and thawed at 37°C. Testing of auto sampler stability of quality control samples indicated that phenytoin would be stable when kept in the auto sampler up to 24 h.

Table 10: Data of stability study

STABILITY DATA			
STABILITY	**NOMINAL CONC. (ng/mL)**	**MEAN OBSERVED CONC. (ng/mL)**	**%CV**
FREEZE THAW (CYCLE 1)	400	424.666	8.331
	4000	4316.666	7.190
FREEZE THAW (CYCLE 2)	400	412.525	3.138
	4000	4119.632	5.593
FREEZE THAW (CYCLE 3)	400	428.758	7.128
	4000	4239.912	6.661
AUTO SAMPLER (24 h)	400	415.910	7.111
	4000	4139.846	9.027

C. DEVELOPMENT AND CHARACTERIZATION OF PHENYTOIN NANOEMULSION

Formulation of nanoemulsion by phase inversion composition (PIC) method (low energy method)

Screening of oil

The choice of an oily phase is often a compromise between its ability to solubilize the drugs and its ability to facilitate formation of nanoemulsion of desired characteristics. Lipophilic drugs are preferably solubilized in o/w nanoemulsion. The ability of the nanoemulsion to remain in the solubilized form is greatly influenced by its solubility in oil. If the solubility is influenced by surfactant and co-surfactant there could be a risk of precipitation. Table… shows the solubility of phenytoin in different oils. Figure11 shows the comparative solubility of phenytoin in different oils.

Table 11: Solubility of Phenytoin in different oils.

Components	Solubility± SD (mg/ml), n=3
Coconut Oil	5±0.57
Sunflower Oil	5±0.84
Plurol Oleique	10±1.57
Vitamin E	15±1.12
Captex GTO	60±0.96
Labrafac	85±1.14

Phenytoin possesses the highest solubility in Labrafac oil (80±5mg/ml). Hence Labrafac oil was selected for the formulation of nanoemulsion. Labrafac is a Medium Chain Triglycerides (MCT). Triglycerides are highly lipophilic and their solvent capacity for drugs is commonly a function of the effective concentration of ester groups, thus on weight basis medium chain triglycerides (MCT)

have higher solvent capacity and resistance to oxidation compared to long chain triglycerides

Selection of surfactant and co-surfactant

The most important problem related to the nanoemulsion based system is the toxicity of the components. Non –ionic surfactants are relatively less toxic than their counterparts and typically have lower CMCs. The surfactant chosen must be able to lower the interfacial tension to a very small value to aid the dispersion process during the preparation of nanoemulsion.

Tween 20, Tween 80 and Solutol HS15 were screened for surfactants. Non-ionic surfactants were used since they are known to be less affected by pH and changes in the ionic strength and are generally regarded as safe and biocompatible. Solubilization of the surfactants and co surfactant with the oil is also important. Co-surfactants are added to obtain nanoemulsion system at low surfactant concentration. Short to medium chain alcohols are added which further reduces the interfacial tension and increases the fluidity of the interface. Alcohols may also increase the miscibility of the aqueous and oily phases due to partitioning between these phases therefore Transcutol, Ethanol, PEG200 and PEG400 were taken for screening of co-surfactant. Cosurfactant can decrease the interfacial tension between the oil and the water in the nanoemulsion and adjust the flexibility of interfacial membrane. Based on the solubility of phenytoin in different cosurfactants, Transcutol P was selected.The right blend of low and high Hydrophilic Lipophilic Balance (HLB) surfactant leads to the formulation of stable nanoemulsion formulation. The surfactant and co-surfactant used in the formulation are Solutol HS15 and Transcutol HP with HLB value of 14 and 4.2 respectively

Table12: Solubility of phenytoin in different surfactants and co-surfactants

Surfactant	Solubility± SD (mg/mL),(n=3)	**Co-surfactants**	Solubility± SD (mg/mL), (n=3)
Solutol HS15	75±1.13	PEG 200	20±1.02
Tween 20	15±0.99	PEG 400	15±1.16
Tween 80	10±1.28	Propylene Glycol	10±0.97
		Transcutol P	110±1.22

Miscibility study

Miscibility study was done by first adding drug in the selected oil and completely solubilizing it. After this surfactant and co-surfactant were added to it and it was vortexed for few minutes. It was then checked if any phase separation, settling or any other physical change. No such change was observed.

Drug-excipients interaction studies

Drug interaction and degradation study was done with the selected oil which showed maximum solubility and with excipients which showed complete miscibility with the selected oil. the results showed no interaction with Labrafac, Solutol HS15 and Transcutol P. The UPLC analysis showed no degradation of phenytoin in the selected excipients as compared to the control and did not show any degradation peak or any physical change

Construction of Pseudo Ternary Phase diagram

Pseudo-ternary phase diagrams of oil, water, and co-surfactant/surfactants mixtures are constructed at fixed cosurfactant/surfactant weight ratios. Phase diagrams are obtained by mixing of the ingredients, which shall be pre-weighed into glass vials and titrated with water and stirred well at room temperature. Formation of monophasic/biphasic system is confirmed by visual inspection. In case turbidity appears followed by a phase separation, the samples shall be considered as biphasic. In case monophasic, clear and transparent mixtures are visualized after stirring; the samples shall be marked as points in the phase diagram. The area covered by these points is considered as the nanoemulsion region of existence. Several methods have been suggested for the preparation of nanoemulsion.

Here some methods are discussed which are freely used for the nanoemulsion preparation.

When 1:1 S_{mix} ratio was used nanoemulsion region obtained was almost negligible. It was same with ratio 1:2. On further increasing the proportion of surfactant in the S_{mix} to 2:1 it was observed that there was an increase in the nanoemulsion region. Maximum amount of the oil that could be emulsified was 9%v/v using 46%v/v of S_{mix}. On further increasing the proportion of surfactant in the S_{mix} to 3:1 ratio there was an appreciable increase in the nanoemulsion region and the maximum amount of the oil that could be emulsified was found to be 12%v/v and 43%v/v S_{mix}. For 4:1 S_{mix} ratio there was further increase in the nanoemulsion region and the maximum amount of the oil that could be emulsified was 15%v/v and 45%v/v of S_{mix} (Fig- 7).

Table 13 shows the percentage composition of the placebo nanoemulsions and Table 14 shows the observation for the thermodynamic stability study of the formulations. Formulations that did not pass the accelerated physical stability tests were dropped out and the remaining were subjected for further studies. Based on the accelerated thermodynamic physical stability studies, two formulations were selected. Table 15 shows the composition of oil, S_{mix} and water of the selected formulation B1 and B4.

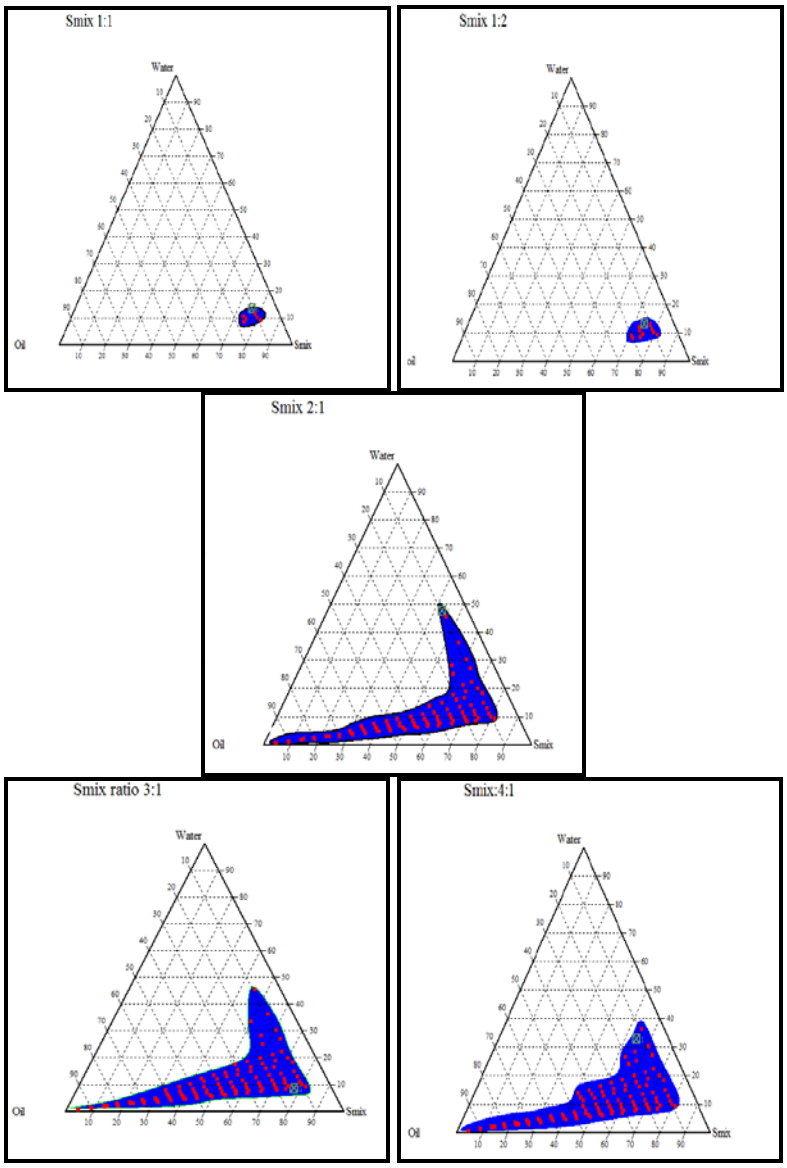

FIG 6: Ternary phase diagrams for different S_{mix} ratios.

Table 13: % Composition of the selected placebo nanoemulsions

FORMULATION CODE	Smix	% Oil	%Smix	% Water
A1		11	45	44
A2		10	40	50
A3		9	36	55
A4		8	32	60
A5		13	47	40
A6	3:01	12	43	45
A7		9	46	45
A8		8	42	50
A9		8	37	55
A10		7	33	60
B1		11	45	44
B2	4:01	15	45	40
B3		14	42	45
B4		13	47	40
B5		12	43	45
B6		9	46	45
B7		8	42	50
C1		9	46	45
C2		8	42	50
C3	2:01	8	37	55
C4		7	33	60
C5		8	47	45

Table 14: Observation for physical stability study of formulations

FORMULATION CODE	**S_{mix}**	**HEATING COOLING CYCLE**	**FREEZE THAW CYCLE**	**CENTRIF-UGATION**	**DISPER-SIBILITY**
A7		X	X	✓	✓
A8	3:1	✓	✓	X	✓
B1		✓	✓	✓	✓
B4	4:1	✓	✓	✓	✓
B6		X	✓	✓	✓
B7		X	✓	✓	✓
C5	2:1	✓	X	X	✓

Table 15: % Composition of oil, S_{mix} and water of the selected formulation

FORMULATION CODE	**Drug (mg/mL)**	**S_{mix}**	**% of oil**	**% of S_{mix}**	**% of water**
B1	5.0	4:1	11	45	44
B4	5.0	4:1	13	47	40

Characterization of nanoemulsion

Refractive index-When the refractive index values for formulations were compared with those of the placebos, no significant ($p>0.01$) differences were found in the values (Table 16) . Therefore it can be concluded that the nanoemulsion formulations were not only thermodynamically stable but also chemically stable and remained isotropic; there were no interactions between nanoemulsion excipients.

Percentage Transmittance

The values of percentage transmittance were found closer to 100% as shown in the Table 16. This indicates that both of the selected formulations are clear and transparent.

Droplet size analysis

Droplet size was analyzed using Malvern zetasizer. Figure 7 and 8 shows the size of the selected formulations. For understanding the behaviour of nanoemulsion, the information on droplet size and size distribution plays an important role since the release and absorption of the drug is related to globule size. Droplet sizes of the selected formulations were determined and the results are shown in Table 16 along with the polydispersity indices.

Polydispersity index is the ratio of standard deviation to the mean droplet size and denotes the uniformity of the droplet size within the formulation. Lower the polydispersity values, higher is the uniformity of the droplet size in the formulation. Both the selected formulations showed lower values and it can be concluded that the droplets are uniformly distributed.

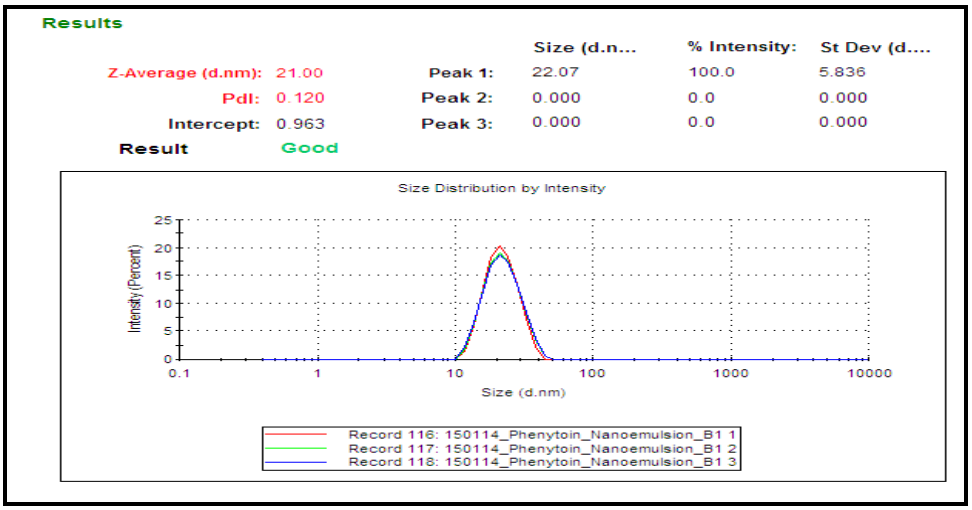

Figure 7: Droplet size distribution of NE B1

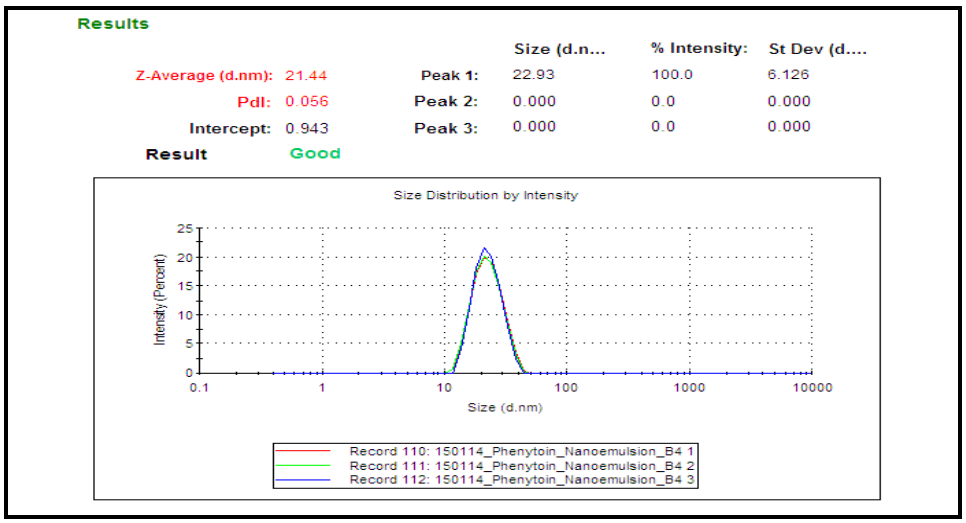

Figure 8: Droplet size distribution of NE B4

Table 16: Table representing Refractive index, %Transmittance, Mean droplet size Zeta potential and polydispersity index of the selected formulations (mean±S.D.)

Formula-tion code	Refractive Index	% Transmittance	Mean Droplet Size (nm)	Polydispe-rsity Index	Zeta potential	Viscosity
B1	1.401±0.001	99.93±0.05	21.00±5.836	0.120±0.0005	-33.7±1.028	138.30±2.15
B4	1.409±0.010	99.76±0.11	21.440±.6.121	0.056±0.002	-30.94±1.63	143.41±1.63

TEM (Transmission Electron Microscopy)

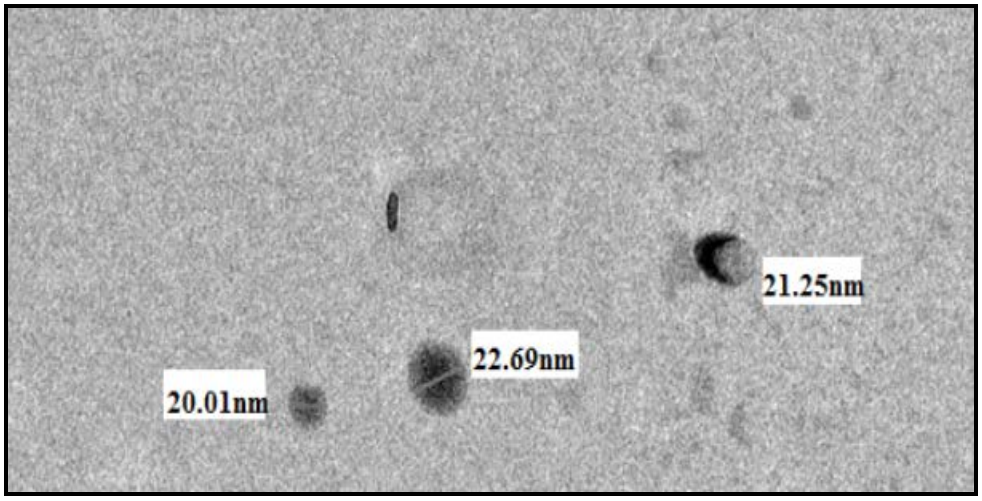

Figure 9: TEM image of formulation NE B1

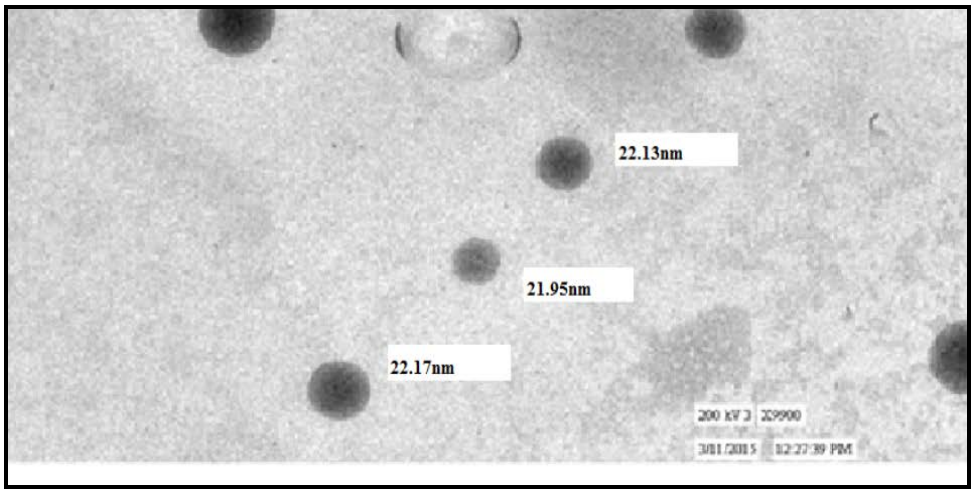

Figure 10: TEM image of formulation NE B4

In TEM positive image, the nanoemulsion appeared dark and the surroundings were bright. Some droplet sizes were measured, as TEM is capable of point to point resolution. TEM image of both the formulations B1 and B4 are shown in Fig 9 and 10. Sizes of the formulations B1 and B4 were found to be ranging between 20-25 nm. The droplet size was in agreement with the results obtained from droplet size analysis using the Zetasizer. As shown in the TEM photograph the diluted nanoemulsion appeared spherical and homogeneous. Most

Viscosity

The viscosity of both the formulations was determined (Table 16). It was observed that the viscosity is directly proportional to the concentration of surfactant used in the formulation.in general it was observed that the viscosity of both the formulations was less.

Zeta Potential

The stability of the colloid system can be predicted in terms of zeta potential. Zeta potential denotes the potential difference between the dispersion medium and the stationary layer of the fluid attached to the dispersed droplet. Negative sign of the zeta potential designate that the formulations were negatively charged and high values of zeta potential indicates the stability of the system. All the successful phenytoin loaded nanoemulsion formulations had zeta potentials between -30-35 mV (Table 16). The presence of free fatty acid esters as contaminants in the oil phases and/or surfactants may be responsible for this negative charge. Thus there are negligible chances of coagulation or flocculation.

***In- vitro* studies**

The formulations that exhibited good thermodynamic stability were subjected to dissolution study. The release of the drug from the formulation was compared and a less significant ($p>0.10$) difference was observed in the drug release from the formulations. The percentage drug release of suspension was found to be comparatively less than that of nanoemulsion. More than 70% of the drug was released in initial 2 h of the dissolution study. This can be correlated with the small globule size of the formulation which provides a larger surface area and thus permitting faster release of the drug. Smaller globules passage through the dialysis bag resulted in instant release whereas drug release in suspension was comparatively low. Dispersibility studies showed that the formulated nanoemulsion is easily and completely dispersed in the medium. The results are depicted in Table 17.

Table 17: Cumulative % of drug released from various formulations.

Cumulative %drug release (Mean ±SD)			
Time (h)	**NE B1**	**NE B4**	**Suspension**
0.25	10.6±1.915	7.8±1.151	6.4±1.150
0.5	31.8±1.853	25.6±1.116	13.8±1.102
1	57.4±1.712	49.2±1.171	25.4±1.136
2	79.1±1.767	70.15±1.369	40.2±1.247
4	85.4±1.924	77.9±1.251	49.4±1.671
8	89.9±1.149	81.8±1.647	55.1±1.105

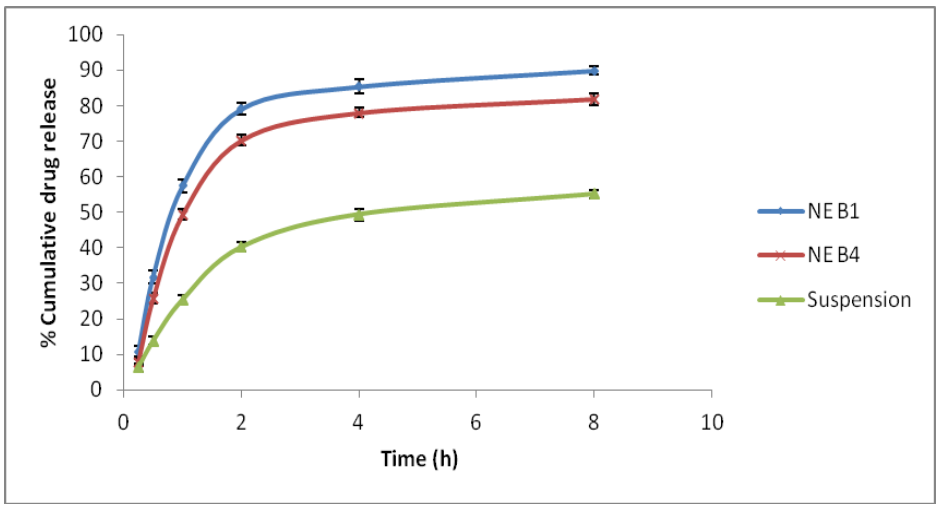

Figure 11: Comparative plot of *In- vitro* release showing cumulative %drug release of various formulations. (Vertical bars represent the standard deviation)

5.8. *In-Vivo* evaluation of nanoemulsion

In this study, to evaluate the enhanced bioavailability following oral administration of optimized nanoemulsion formulations (NE-B1 and NE-B4) and drug suspension were administered to conscious mice. Plasma samples were analyzed using LC-MS-MS on the basis of the already developed and validated bioanalytical method in Part- A. Table 18 and 19 shows the plasma concentration of phenytoin following oral administration of optimized nanoemulsions and table 17 shows the plasma concentration of suspension. These results clearly showed that there were higher plasma concentrations when administered orally in the form of nanoemulsions as compared to the suspension. The maximum plasma concentration was observed after two hours of administration of optimized formulations. C_{max}, T_{max}, AUC for the suspension, NE-B1 and NE-B4 was found to be 3.5 µg/mL, 2.05 hr, 33.5 µg.h/mL ; 14.6 µg/mL, 3.0 hr, 475.25 µg.h/mL and 22.5 µg/mL, 2.0 hr, 93.05 µg.h/mL.

Pharmacokinetic parameters revealed that the extent of oral absorption and hence oral bioavailability of phenytoin was enhanced with nanoemulsion compared with that of suspension. Phenytoin administration in Labrafac oil nanoemulsion showed a significantly higher ($p<0.01$) C_{max} and AUC in plasma. The small droplet size, and hence the large surface area might have contributed to the increased bio availability.

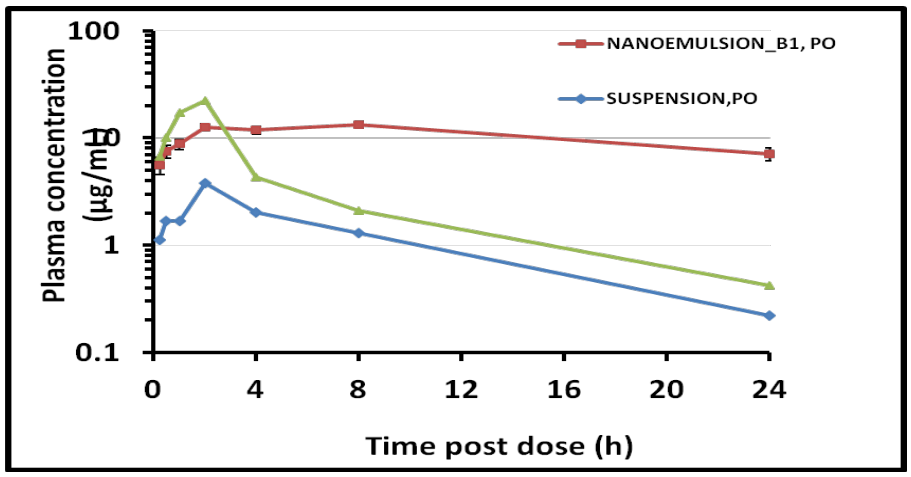

Figure 12: Plasma concentration time profile of nanoemulsions and suspension.

Comparison of pharmacokinetic parameters in the plasma showed higher C_{max} and AUC values when phenytoin was administered in the nanoemulsion as compared to the suspension and shows the potential role of developed formulations. Thus bioavailability enhancing capacity of nanoemulsion could be successfully proven. Thus the developed nanoemulsion can serve as a dose adjustable anti-epileptic formulation, particularly useful for pediatric population.

REFERENCES

1. Abbaspour, A., Mirzajani, R., 2005. Simultaneous determination of phenytoin, barbital and caffeine in pharmaceuticals by absorption (zero-order) UV spectra and first-order derivative spectra—multivariate calibration methods. Journal of Pharmaceutical and Biomedical Analysis; 38: 420-7.
2. Bakshi, M and Singh, S., 2002.Develpoment of validated stability indicating assay method –critical review.Journal of Pharmaceutical and Biomedical Anlaysis; 28:1011-40
3. International Conferemce on Harmonization (ICH), Q2 (R1), Validation of analytical procedures: text and methodology,2005.
4. Mulsa, N., Sanghvi, G., Purohit, P., Sheth, N., Vaishnav, D., 2013. Development of the UV Spectrophotometric Method of Phenytoin Sodium in API and Stress

Degradation Studies. Pharm Analysis & Quality Assurance; 1: 1-5

5. Kumar, K., Saini, G., Nair, A., Sharma, R., 2012. UPLC: A Preeminent Technique In Pharmaceutical Analysis. Acta Poloniae Pharmaceutica - Drug Research; 69:371-80.

6. Pandey, S., Yadav, A.K., Singh., 2012. Bio-Analytical Method Development And Its Validation For Estimation Of Phenobarbital In Human Plasma Using Liquid Chromatography Coupled With Tandem Mass. International Journal of Pharmacy and Pharmaceutical Sciences; 4(5): 288-92

7. Queiroz., 2008. Quantification of carbamazepine, carbamazepine-10,11-epoxide, phenytoin and phenobarbital in plasma samples by stir bar-sorptive extraction and liquid chromatography. Journal of Pharmaceutical and Biomedical Analysis; 48: 428–34

8. Acharya , S.P., Pundarkakshudu, K., Upaddhyay, P., Shelat, P., Lalwani, A., 2015. Development of phenytoin intranasal microemulsion for treatment of epilepsy. Journal of Pharmaceutical Investigation; 15:190-93.

9. Wanga, X., Jianga, Y., Wanga, Y.W., Huangb, M.T., Hoa, C.T., Huanga, Q., 2008. Enhancing anti inflammation of cucrcumin through oil in water nanoemulsions. Food Chemistry; 108(2):419-24.

10. Mason, T.G., Meleson, K., Wilking, J., Chang, C.B., Graves, S.M., 2006. Nanoemulsion: Formation, structure and physical properties, J. Phys.: Condens. Matter. 18, R635-R666.