Chapter 4

Experimental

4. Experimental

4.1 Materials and Instruments used

Folic acid, EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide), Gelatin Type A, (Bloom Strength 175-225) were purchased from Sigma-aldrich chemicals private limited. Phenytoin Sodium USP was a gift sample. Dialysis membrane (Molecular weight cutoff between 12000-14000 Da) was purchased from Himedia, India. All other chemicals were of analytical/ HPLC grade. Instruments used and their make-up are listed in **Table 4.1**.

Table 4.1. Instruments used and their make-up

NMR Spectroscopy	Agilent 500/54/AR spectrometer
IR Spectroscopy	Perkin Elmer Spectrum One FT-IR spectrometer
Gas Chromatography Head Space (GCHS)	Agilent 7890A
DSC	Perkin Elmer DSC 6000, software- Pyris Manager
SEM (Field emission SEM, FESEM)	Nova Nano SEM 450,
SEM Coater	Jeol JFC-1600 Autofine Coater
XRD	PANalytical X'pert Powder
UV-Vis	Perkin Elmer UV/Vis Spectrometer lamda 35, software UV WINLAB
HPLC	Dionex UPLC Ultimate 3000, software- Chromeleon
Centrifuge	Rota 4R-V/FM Plasto Craft

4.2 Synthesis of Gelatin Folate

Gelatin-folate was synthesized by using EDC chemistry (Pu et al., 2009 & Nakajima and Ikada, 1995). Briefly, Folic acid was activated by allowing it to react with a 14-fold excess of aqueous EDC solution at cold temperature (below 5°C) for 4 hours. Aqueous solution of gelatin was added dropwise to the FA-active ester solution and stirred overnight at room temperature. Acetone was added to precipitate the gelatin-folate conjugate formed, and precipitate was separated by filtration.

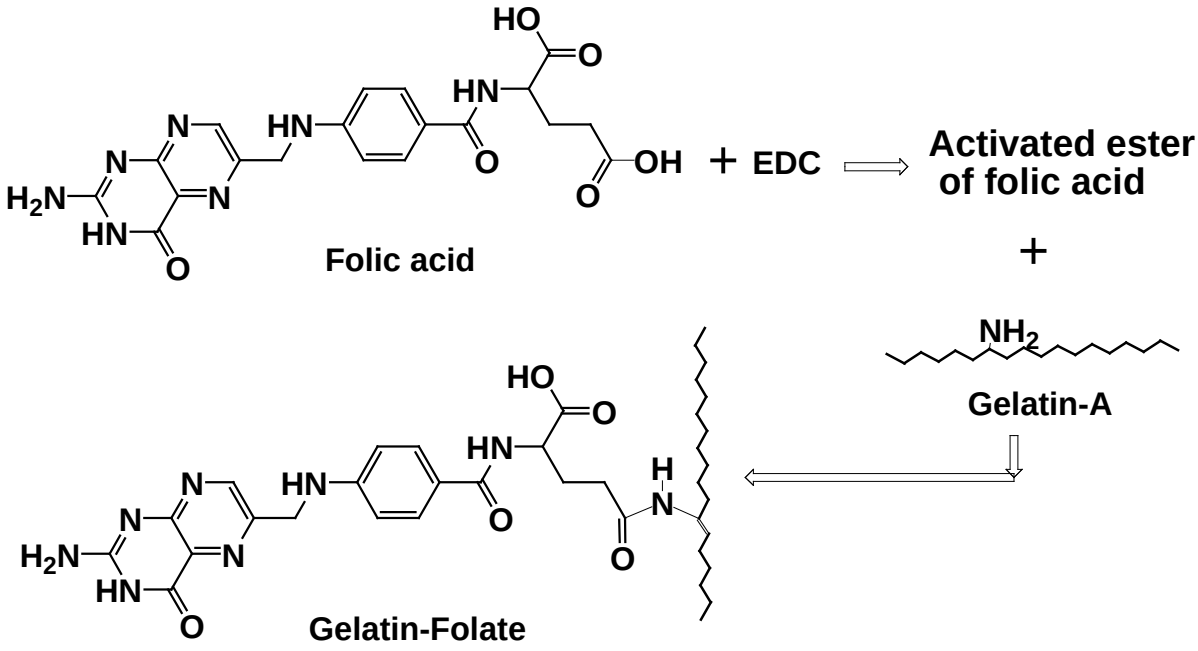

Figure 4.1. Scheme: Conjugation of folic acid to gelatin

4.2.1 Characterization of gelatin folate

4.2.1.1 Nuclear Magnetic Resonance Spectroscopic (NMR) analysis

The ^{1}H NMR and ^{13}C NMR spectra of folic acid, gelatin, and gelatin- folate were recorded in D_2O on Agilent 500MHz FTNMR spectrometer. The ^{1}H NMR and ^{13}C NMR chemical shifts (δ) were reported as parts per million (ppm).

4.2.1.2 Fourier Transform Infra Red (FTIR) analysis

Infrared spectra were recorded on a Perkin-Elmer Spectrum One FT-IR spectrometer. Prior to analysis, samples were prepared by mixing 1:5 of sample: KBr (w\w) followed by punching the powder into a thin disc. Spectra were obtained between 4000–400 cm^{-1}.

4.2.1.3 Differential Scanning Calorimeters (DSC) Analysis

DSC thermograms were recorded on a Perkin Elmer DSC 6000. Approximately 5 mg of particles were loaded onto standard aluminum pans. The analysis was carried out at a temperature heating rate of 10 °C/min and a temperature range of 30–400 °C.

4.2.1.4 X-ray Diffraction (XRD) Analysis

X-ray diffraction patterns were recorded on a PANalytical X'pert Powder diffractometer using Ni-filtered, CuKα radiation (λ=1.5418Å), a voltage of 45 kV and a current of 25 mA. The samples were recorded over the most informative range from 3° to 90° (60°) of 2θ, with a step time of 2s and a step size of 0.02°.

4.2.1.5 Morphological characterization using SEM

SEM micrograms were recorded on a Nova Nano SEM 450 microscopy, by coating the sample with gold prior to the microscopic examination using ion sputtering. The accelerating voltage was kept at 20 KV.

4.3 HPLC analytical method

Determination of phenytoin sodium from invitro samples was done as described previously as per USP official monograph of phenytoin sodium. Detection was made at 220 nm using PDA detector. Monobasic ammonium phosphate buffer 0.05 M (pH 2.5 with phosphoric acid): Acetonitrile: Methanol and water in the ratio of 45:35:20 was used as mobile phase for HPLC system. This mixture was filtered through 0.45 μm nylon filter and degassed before injection. Mobile phase itself was used as diluents for standard and test preparation. Stock solution of Phenytoin sodium was prepared by dissolving 10 mg of drug in 100 ml of mobile phase. This stock solution was appropriately diluted with mobile phase to obtain 100, 50, 25, 12.5, 6, 3, 2, 1 μg/ml concentration of drug.

4.3.1 Chromatographic system:

- Detector: PDA detector
- Reversed phase C18 column (5μ; 250 × 4.6 mm)
- Column temperature: 40°C
- Flow rate: 1 ml/min
- Injection volume: 20 μl
- Run time: 15 min

4.3.2 Linearity

Linearity tests were performed in the concentration range of 100 to 1 μg/ml. Calibration graphs were constructed by plotting peak-area of phenytoin as ordinate (y) verses nominal concentration in μg/mL as abscissa (x). The calibration plot was fitted by robust least square fitting using 1/X2 weighing. The linearity of the relationship between peak area and phenytoin concentration is demonstrated by the correlation coefficients or regression (r) obtained from the equation 'y = mx + b'.

4.3.3 Accuracy and precision

Accuracy and precision was evaluated by spiking phenytoin into control plasma at three concentration levels: 25, 50, and 100 μg/mL for phenytoin determination. The samples were analyzed with proposed HPLC method for three replicates within the same day and three consecutive days for the intra-day and inter-day accuracy and precision, expressed as relative error (RE) % values and RSD % values, respectively.

$$\text{Recovery or Relative Error (RE)}\% = \frac{\text{Concentration Detected}}{\text{Concentration Spiked}} \text{ x } 100$$

$$\text{Relative standard deviation (RSD) or Coefficient of variation (CV) } \% = \frac{\text{Standard deviation of concentration detected}}{\text{Mean concentration detected}} \text{ x } 100$$

4.4 Fabrication of Gelatin /Gelatin folate nanoparticles

4.4.1 Selection of fabrication methods

1) **Single step desolvation method:** Gelatin nanoparticles (GNPs) were prepared by single step desolvation process as described previously (Ofokansi et al 2010). In principle, Gelatin was dissolved in distilled water under constant heating at 40 ± 1°C, The pH of the gelatin solution was adjusted between 4 and 5. To the above aqueous polymer phase, DPH Sodium was added in, and followed by dropwise addition of acetone as desolvating agent to form GNPs. At the end of the process, glutaraldehyde solution (25% v/v aqueous solution) was added as a cross-linking agent, and the solution was stirred for 6 hours at 1000 rpm.
2) **Two step desolvation method:**

   Gelatin nanoparticles were prepared by a two step desolvation process as described previously 9Coester et al 2000 & Goswami et al 2010). Gelatin was dissolved in distilled water under constant heating at 40 ± 1°C. Acetone was added to the gelatin solution as a desolvating agent to precipitate the high-molecular-weight (HMW) gelatin. The supernatant was discarded, and the HMW gelatin was redissolved in distilled water with stirring at 600 rpm under constant heating. The pH of the gelatin solution at the second desolvation step was adjusted between 4 and 5. To the above polymeric solution, DPH Sodium was added in, and followed by dropwise addition of acetone as desolvating agent to form GNPs. At the end of the process, glutaraldehyde solution (25% v/v aqueous

solution) was added as a cross-linking agent, and the solution was stirred for 6 hours at 1000 rpm.

4.4.2 Optimization of formulation variables

Method of fabrication of nanoparticles

Volume of acetone

Percentage of polymer

Degree of cross-linking

Duration of crosslinking

Optimization of method of fabrication of nanoparticles and volume of acetone were carried out as per the preliminary batches listed in **Table 4.2** and remaining variables as per the secondary batches listed in **Table 4.3**

Table 4.2. Composition of preliminary batches

Methods	**Batch Code**	**Gelatin (%)**	**Acetone (ml)**	**GA Conc.**	**Ratio of Drug to Polymer**
One Step Desolvation method	SS1	2	15	$15x10^{-3}$	1: 2
	SS2	2	20	$15x10^{-3}$	1: 2
	SS3	2	25	$15x10^{-3}$	1: 2
Two Step Desolvation method	TS1	5	7.5	$15x10^{-3}$	1: 2
	TS2	5	10	$15x10^{-3}$	1: 2
	TS3	5	12.5	$15x10^{-3}$	1: 2
	TS4	5	15	$15x10^{-3}$	1: 2

Table 4.3 Composition of Selected batches

Batch Code	**Gelatin (%)**	**Conc. of GA (mg/ml)**	**Duration of Crosslinking (hr)**	**Ratio of Drug to Polymer**
GT1	3	$15x10^{-3}$	6	1:3
GT2	4	$15x10^{-3}$	6	1:3
GT3	5	$15x10^{-3}$	6	1:3
GT4	6	$15x10^{-3}$	6	1:3
GT5	7	$15x10^{-3}$	6	1:3
GA1	5	$5x10^{-3}$	6	1:3
GA2	5	$10x10^{-3}$	6	1:3
GA3	5	$20x10^{-3}$	6	1:3
DC1	5	$15x10^{-3}$	4	1:3
DC2	5	$15x10^{-3}$	8	1:3
DR1	5	$15x10^{-3}$	6	1:2
DR2	5	$15x10^{-3}$	6	1:4
GF1	5*	$15x10^{-3}$	6	1:3

***** Gelatin Folate. Nanoparticles of gelatin folate were formulated according to the composition of best batch of gelatin nanoparticles, GT3.

4.4.2.1 Volume of desolvation

The influence of volume of acetone in both first and second step of desolvation process was studied by preparing nanoparticles with a range of volume of acetone. 5% aqueous gelatin solution was desolvated with 7.5, 10.0, 12.5 and 15.0mL of acetone, respectively. These prepared nanoparticles were studied for their particles size and stability. In the first desolvation step, the volume of acetone of second desolvation step was kept constant, i.e.

20 mL. The influence of volume of acetone in the second step of desolvation process was studied with 10.0, 15.0, 20.0, 25.0 mL of acetone, wherein 10.0 mL of acetone was kept constant for the first desolvation step.

4.4.2.2 Particle size, PDI, Zeta potential

Particle size, polydispersity index (PDI) and surface potential (zeta potential) of the formulated gelatin nanoparticles were measured by Photon Correlation Spectroscopy (PCS) using NanoZS, Malvern Instrumnents, UK, based on dynamic light scattering technique.

4.4.2.3 Total drug content and Drug loading (DL %):

The total amount of phenytoin sodium present in the given amount of nanoparticles was estimated as follows:- Accurately weighed 100 mg of phenytoin sodium was dissolved in small volume of buffer and sonicated to dissolve it, and the volume was made up with the same diluent. After filtration through a 0.45µm membrane filter, the amount of drug presence was estimated by HPLC method developed using UV detector at its λ^{max} - 220 nm. The filtered solution from the placebo nanoparticles (without drug) was taken as blank.

Percentage of drug loading (DL %) was calculated according to the following equation:

$$DL\% = \frac{WD}{WT} \times 100$$

Where, DL- Drug Loading, W_D – Weight of the drug in nanoparticles, W_T – Total weight of the nanoparticles.

4.4.3 In vitro release studies

In vitro release kinetics was evaluated by diffusion through dialysis membrane. Briefly, Dialysis membrane (molecular weight: 12,000 Da) was soaked in milliQ water for 12 h before experiment. The membrane was tied to one end of a both-end opened dialysis tube (Franz diffusion cells) and accurately weighed nanoparticles equivalent to 25 mg of phenytoin sodium in small volume of phosphate-buffer pH 6.8 was added into the tube. The tube was immersed into a beaker containing 200 ml of phosphate-buffer pH 6.8, with the help of a burette stand clamp. This whole assembly was kept on a magnetic stirrer to maintain temperature at 37±0.5°C and stirring at 50 rpm. At fixed interval, samples were withdrawn from receiver compartment and same volume of dissolution media was replaced by fresh medium, and sink condition was maintained. Cumulative amount released was calculated using the following relationship,

$$\text{Cumulative amount released } \% = \frac{\text{Amount presence}}{\text{Actual amount}} \text{ x } 100$$

4.4.4. Drug release mechanism

The order of drug release from DPH-NP and DPH-NP-FA was studied by fitting the release profile data into Zero order, First order and Higuchi model kinetics; and further, the mechanism of drug release was studied through Korsmeyer–Peppas model for the first 60% of the total amount of drug released.

Korsmeyer–Peppas equation,

$$\frac{Mt}{M\infty} = Ktn$$

Where, Mt/M∞ is the fractional drug release into the dissolution medium, K is the rate constant and 'n' is the diffusional exponent, which characterizes the drug release mechanism. Interpretation of the drug release mechanism was according to **Table 4.4**

Table 4.4 Interpretation of diffusional release mechanisms from polymeric films

Release exponent (*n*)	**In case of microsphere**	**Drug transport mechanism**	**Rate as a function of time**
0.5	0.43	Fickian diffusion	$t^{-0.5}$
0.5<*n*<1.0	0.43<*n*<0.85	Anomalous transport	t^{n-1}
1.0	0.85	Case-II transport	Zero order release
Higher than 1.0	Higher than 0.85	Super Case-II transport	t^{n-1}

Adopted from Costa and Lobo, 2001.

4.4.5 Characterization of DPH-NP

Infrared spectra of phenytoin sodium, glutaraldehyde, native gelatin, gelatin folate, gelatin nanoparticles and gelatin folate nanoparticles were recorded on a FTIR-8400S-Shimadzu-spectrophotometer. For Solid state characterization, DSC and XRD were recorded on Perkin Elmer DSC 6000 calorimeter and PANalytical X'pert Powder diffractometer, respectively. SEM micrograms were recorded on a Nova Nano SEM 450 microscopy for morphological characterization.

4.5 Determination of Residual Solvents by GCHS

4.5.1 Solutions

4.5.1.1 *Standard stock solution*

5.0 ml standard stock solution was made up to 100 ml with *N*,*N*-Dimethylformamide.

Table 4.5. Name of the standard solvent and weight taken

Name of solvents	**Batch No.**	**Purity (%)**	**Weight (mg)**	**Limit (ppm)**
Methanol	SE2SF62327	99.7	243.33	3000
Ethanol	K42133227	99.9	407.64	5000
Diethylether	L04A-0404-1512-13	99.0	414.33	5000
Acetone	I624822207	99.9	401.46	5000
Isopropyl alcohol	05028	99.8	412.36	5000
Acetonitrile	SK2SF62753	99.8	36.43	410
Dichloromethane	K43243344216	99.9	59.91	600
n-Hexane	60810	99.0	25.92	290
Ethyl acetate	IJ9IF90325	99.7	402.20	5000
Tetrahydrofuran	I643701225	99.9	58.08	720
Cyclohexane	NL18816005H2	99.8	307.96	3880
n-heptane	9343 6905-3	99.5	405.88	5000
n-Butanol	606263027	99.5	401.87	5000
Methyl isobutyl ketone	3BP830038	98.0	413.84	5000
Toluene	K40606825	99.9	77.02	890

4.5.1.2 *Preparation of blank vial*

5.0 ml of *N*,*N*-Dimethylformamide in a head space vial with screw cap.

4.5.1.3 *Preparation of standard vial*

5.0 ml of standard solution in head space vial with screw cap.

4.5.1. 4 *Preparation of test vials*

Test vials were prepared as per **Table 4.6** in 5 ml of *N*,*N*-Dimethylformamide.

Table 4.6 Test samples and weight taken

Name of the sample	**Weight taken**
Folic acid	0.20089 gm
Gelatin	0.20029 gm
Gelatin folate	0.20149 gm
Phenytoin sodium	0.20056 gm
Formulation 1	0.20001 gm
Formulation 2	0.20025 gm

The blank, standard and test samples were placed separately in a head space vial with screw cap, each containing 5 ml of *N*,*N*-Dimethylformamide.

4.5.2. Chromatographic System

Table 4.7 Chromatographic System of GCHS

Instrument	Agilent 7890A
Column	DB-624, (30 m x 0.25 mm x 1.4 µm), capillary column.
Temperature	
Column	Temperature at 40°C for 10 minutes, then raised to 130°C at a rate of 8°C per minute and maintained for 5 minutes, then raised to 240°C at rate of 35°C per minute and maintained for 15 minutes.
Incubation	At 80°C for 20 minutes and agitator speed set for 500 rpm.
Inlet port	At 200°C
Detector	At 270°C.
Carrier gas	1. Nitrogen at a rate of 0.5 ml per minute. 2. Hydrogen gas at a rate of 400 ml per minute
Injection volume	About 500 µl
Split ratio	25 :1
Run time	45 minutes.
Detector	Flame-ionization detector

4.5.3 Calculation of residual solvents (ppm).

$$Residual\ solvents\ (ppm) = \frac{Area\ of\ sample}{Avg.area\ of\ std} \times \frac{Dilution\ of\ std}{Dilution\ of\ sample} \times Purity\ of\ Std \times 10000$$

4.6 Pharmacodynamic and Pharmacokinetic studies

4.6.1 Animal Study protocol

CPCSEA Guidelines were followed during the animal handling and these studies had the approval of the Institutional Animal Ethical Committee (IAEC). No. Dean/ 10 – 11/ 234, dated 21.08.2010. Male Wurster rats were used for both pharmacodynamic and pharmacokinetic studies. Rat weighing 100 to 120 gm were selected and housed in a temperature-controlled (23 ± 2ºC) and humidity-controlled (65%) room on 12:12-h light/dark cycle (lights on 08:00±20:00 h) for at least one week.

4.6.2 Pharmacodynamic studies through *in vivo* method

4.6.2.1 Maximal electroshock method

Rats were divided into four groups:- 1, DPH suspension (std); 2, DPH-NP; 3, DPH-NP-FA; 4, Vehicle (control). All the rats were randomly assigned to experimental groups. Each group except control was further divided into four subgroups (n=6).

All the animal groups (DPH suspension, DPH-NP and DPH-NP-FA) except control group are treated with 4, 8, 12, and 16 mg/kg DPH or formulation containing equal amount of DPH; control group was treated with vehicle only.

At 30 min after the intraperitoneal (i.p) injection of DPH suspension (std), DPH-NP, DPH-NP-FA and control (0.9% W/V normal saline) maximal electroshock (MES) was induced in rats by stimulating them with electrodes placed on both eyes connected to an electroshock apparatus. The supramaximal corneal electroshock currents of 150 mA (five

to seven times of threshold current) was provided for 0.25 sec duration. % incidene was calculated from inhibition of THE (*Tonic Hindlimb Extension)* component of MES or from its complete disappearance (Yende et al., 2009 & Hasebe et al., 2010). ED50 value of DPH suspension (std), DPH-NP, DPH-NP-FA was calculated from % incidence. ED50 value is a dose of a drug necessary to protect 50% of the experimental animals against seizure induced.

4.6.3 Bioanalytical HPLC Method

Determination of phenytoin sodium in biological samples were as per the procedure recorded under the section 4.3. However, to calculate the percentage of drug loss during liquid-liquid extraction step, calibration curve was constructed by adding phenytoin sodium in the concentration range of 100 to 1 μg/ml before (extracted) as well as after (spiked) the liquid-liquid extraction step.

4.6.4 Pharmacokinetic studies

Animals were divided into four groups (n=20). Rats were fasted overnight before oral dose administration and approximately 3 h post-dose, but had free access to water. The control group received blank vehicle, and others groups received phenytoin suspension or formulation equivalent to 25 mg/kg (in 0.9% W/V normal saline in a volume of 2 ml/kg), per oral via using a ball-tipped needle.

4.6.4.1 Collection of samples

At each interval, three animals from each groups were decapitated and blood samples (whole available quantity after decapitation) from trunk and tissues of interest were collected in eppendrof tubes. Blood was collected in eppendrof tubes containing about 20µl heparin each. Blood samples and Tissues of interest i.e, brain were collected immediately decapitation at different time points and they were blotted dry with tissue paper. Blood and tissue samples were frozen at -20°C until analysis.

4.6.4.2 Preparation of samples

On the day of analysis, blood samples as well as tissue samples were allowed to thaw. Plasma was obtained by centrifugation at 5000×g for 10 min at 4°C. Plasma proteins were then precipitated by adding 1200 µl of acetonitrile to 600 µl aliquot of the plasma (2:1). After rigorous vortex-mixing for 1min, the mixtures were centrifuged at 15000×g for 15 min at 4°C. then, 600 µl of mobile phase was added to 1200 µl aliquot of the supernatant (1:2) and centrifuged at 15000×g for 7 min at 4°C.

In case of brain samples, they were allowed to thaw, washed with saline; and weighed prior to homogenization in 0.5 ml normal saline. Tissue samples were cooled on ice after the homogenization procedure; the homogenate was then centrifuged at 21,000×g for 10 min. then, the homogenate was in the same way the plasma samples were treated. An aliquot of supernatant was then transferred to the HPLC sample vial and 20 µl was injected into the HPLC system for analysis with an autosampler. Drug-free plasma and

tissue samples obtained from control group were treated in the same way. Spiked and extracted calibration curve were obtained from them.

4.6.5 Pharmacokinetic analysis

Pharmacokinetic parameters were calculated by noncompartment model using Microsoft excel through PK Function Add on. The plasma phenytoin concentration versus time profiles were used to determine maximum plasma concentration (Cmax), time to achieve maximum plasma concentration (Tmax), area under the curve ($AUC_{0.5–12h}$), half-life ($t_{1/2}$). $AUC_{0.5–12h}$ was determined by linear trapezoidal summation.

4.6.6 Statistical analysis

One Way ANOVA with Newman-Keuls post test (to compare all pairs of column) was used to test the significance of differences between pharmacokinetic parameters of std and test formulation treated groups by GraphPad prism software version 5.0, and the adopted level of significance is $P<0.05$.