4. Materials and Methods

4.1. Materials

Berberine chloride was gift sample obtained from Inga Pharmaceuticals, Mumbai, India. Sodium lauryl sulphate (SLS), Polysorbate 80 (T-80), Pluronic F-68 (F-68) and Cetyl trimethyl ammonium bromide (CTAB) were kind gifts from BASF and Tatva Chintan Pharma Chem Pvt Ltd., Ankeleshwar, India respectively. PCL (MW ~45 000 g/mol) was procured from Sigma Aldrich, USA. Dialysis membranes (molecular weight cutoff between 12,000 and 14,000) were purchased from Hi-Media, Mumbai, India. Nanosep® Centrifugal filter devices (Omega Membrane, MWCO 100 kD) were purchased from Pall Life Sciences Mumbai, India. The water used in all experiments was ultrapure, obtained from a Millipore–DirectQ UV® ultra pure water system Millipore, France. Other chemicals of analytical grade were purchased locally and used as received.

4.2. Methods

4.2.1. HPLC analytical method development

A reverse phase - High Performance Liquid Chromatography (HPLC) analytical method was used for quantification of BBR (Zeng, 1998). The HPLC system consisted of a 515 HPLC binary pump (Waters, USA), a Rheodyne 7725i manual injector (Waters, USA), Photodiode Array Detector (Waters, USA) and an operating software Empower Node 2054. Drug quantification was carried out using a C_{18} reverse-phase (250 × 4.6

mm, 5 mm) ODS2 column (Waters Corp., Milford, MA, USA). The mixture of acetonitrile and 0.02 M phosphoric acid in the ratio of 45:55 v/v was used as mobile phase. The mobile phase was filtered through a 0.45µm millipore filter and degassed prior to use. The flow rate was 1.0 ml/min. Detection was performed at a wavelength of 346 nm (λ max) and the column was maintained at a constant temperature (25 ± 1 °C). Standard curves were constructed in the range of 2 – 10 µg/ml (triplicate) and validated for linearity, precision, accuracy, limit of quantification (LOQ) and limit of detection (LOD).

4.2.2. Solubility study in aqueous, different pH solutions and surfactants

The solubility of BBR was determined in different buffers and Millipore water (MPW) as a function of pH at 25°C. Buffers such as pH 1.2 (hydrochloric acid), pH 3.0 (acid phthalate), pH 4.5 (neutralized phthalate), pH 6.8 and pH 7.2 (phosphate) were prepared according to USP. Briefly, the excessive amount (300 mg) of BBR was added in six conical flasks each containing 20 ml of water and respective pH 1.2 to 7.2 buffer solutions. The flasks were tightly corked, placed in a thermo stated water bath at 25°C and agitated at 70 rpm for 24 h. After this period, separated aliquots from each flask were filtered through 0.45µm filter then diluted with respective solutions and analyzed using HPLC. Each

solubility value was determined in triplicate (n=3) and the results were reported as mean ± standard deviation (SD).

Drug solubility was also screened in the selected buffer solution with various surfactants for better understanding of solubility behavior of BBR in their presence. The various surfactants such as SLS (anionic), CTAB (cationic), T-80 and F-68 (non-ionic) were employed in the concentration of 10 - 50 mM. Media was prepared with various surfactants at their respective concentrations in fresh buffer.

4.2.3. Development of nanoformulations

4.2.3.1. Optimization of process variables

In this study, effect of different process variables such as needle size (diameter), injection rate, stirring rate on the particle size was evaluated. Placebo nanoparticles (without drug) were prepared by nanoprecipitation method (Figure 4.1.). The volumes of aqueous phase (50 ml), surfactant concentration, Pluronic F-68 (50 mM), temperature (25°C) were kept constant for all batches. Total 8 batches were prepared as shown in Table. 4.1.

4.2.3.2. Optimization of formulation variables

The optimization of formulation variables were carried out using 3^2 factorial design. The experimental design was created using Design-Expert software (Trial version 8.0.7.1, Stat-Ease Inc., Minneapolis, MN, USA) (Chakraborty *et al*., 2013). Response surface quadratic model was

performed for optimization of formulation variables. Two independent formulation variables such as drug to polymer ratio (A) and concentration of stabilizer (B) each at three coded levels were designed and shown in Table 4.2. The effect of these factors was studied on the particle size (PS) (Y_1), polydispersity index (PDI) (Y_2), and entrapment efficiency (EE) (Y_3) of the formulation as response variables. The order of the experiments was randomized to avoid experimental bias. All other parameters (temperature, injection rate, stirring rate, evaporation time, volume of organic and aqueous phase) were kept constant to minimize fluctuations. The formulation and the response variables are mentioned in Table 4.3. Contour plots and three-dimensional (3D) response surface graphs were generated for diagrammatic depiction of the values of the response. They are supportive in explaining the relationship between independent variables and responses. Statistical analysis of data was done on the basis of ANOVA provided in the software.

4.2.3.3. Preparation of BBR-NP

Nanoparticles were prepared by using nanoprecipitation method (Singh and Muthu, 2007). In brief, the polycaprolactone (PCL) was dissolved in acetone (organic phase) at 40°C and added to ethanolic solution of BBR. The organic phase was dropped into the stabilizer solution (aqueous phase) using glass syringe equipped with specific size of needle at the optimized rate directly under optimized magnetic stirring at 25°C. The

acetone was evaporated at 40 °C under reduced pressure, using a Rotary evaporator (IKA® RV 10 digital, Germany). The obtained nanodispersion was centrifuged at 15000 rpm for 30 min and obtained nanoparticles pellet was rinsed thrice with water. Finally, pellet was lyophilized using lyophilizer (Lypholizer, Decibel, India) for 36 h at -40°C. The lyophilized nanoparticles were stored in desiccators until further use.

4.2.3.4. Preparation of BBR-SCNP

The surface coating of the BBR-NP was carried out as method described by Kulkarni and Feng (Kulkarni and Feng, 2013). The BBR NP was mixed and incubated in 1% w/v aqueous solution of vitamin E TPGS for overnight at room temperature. The surface coated nanoparticles were separated by centrifugation at 15,000 rpm for 30 min at 4°C (Remi, India). The obtained surface coated nanoparticle pellets were lyophilized using lyophilizer (Lypholizer, Decibel, India) for 36 h at -40°C. Lyophilized nanoparticles were stored in cool condition (8°C) until further use.

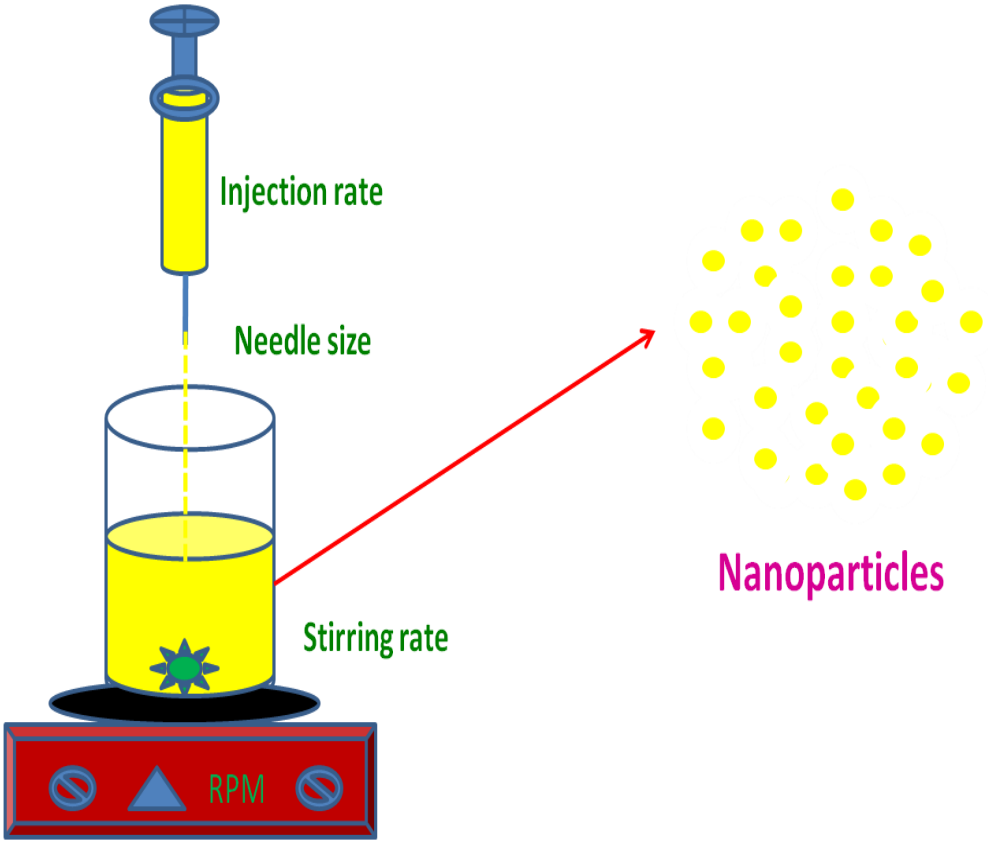

Figure 4.1. Schematic diagram of nanoprecipitation method

Table 4.1. Effect of process variables on particle size of placebo nanoparticles

Parameter	Level	
	Low	High
Stirring rate (rpm)	600	1200
Injection rate (ml/min)	6	12
Needle size (No/ i.d)	20 (0.90 mm)	26 (0.45 mm)

Table 4.2. Factorial design parameters and experimental conditions

Translation of coded levels in actual units			
Coded levels	-1	0	+1
A: Drug to polymer ratio	1:2	1:3	1:4
B: Stabilizer concentration (mM)	10	25	50

Table 4.3. Formulations of the nanoparticles using 3^2 factorial design

Run	**Formulations**	**A**	**B**
1	F-1	1:4	50
2.	F-2	1:3	25
3.	F-3	1:3	10
4.	F-4	1:2	25
5.	F-5	1:2	10
6.	F-6	1:3	50
7.	F-7	1:4	10
8.	F-8	1:4	25
9.	F-9	1:2	50

A: Drug to polymer ratio and B: Stabilizer concentration (mM)

4.2.4. Development of Naplet Technology

4.2.4.1. Preparation of Naplet

The Naplet was prepared manually using an in house laboratory developed method by hand press device made up of stainless steel as shown in Figure.4.2. Usually, the device has used in preparation of South Indian traditional snack items and commercially available in Indian markets. However, this device has first time employed in pharmaceutical research for delivery of drug as a Naplet. The device is having two parts i.e. holder and plunger. The holder (feed chamber) part is a hollow stainless tube typically open at two side end. The plunger part is a piston rod that passes inside the holder of naplet. The approximate length of holder is 15 cm and diameter is 4 cm. The smooth damp mass was prepared by uniform mixing of pre weighed excipients with BBR-NP dispersion as mentioned in Table. 4.4. The prepared damp mass was carefully loaded in holder part and allowed to pass through lower side orifice by gentle press of side spring hand of naplet device. The wet extruded product (naplet) was placed on filter paper and made in to pieces (approx. 4 mm width) with sharp knife. Finally, the pieces were allowed for drying overnight at room temperature.

4.2.4.2. Optimization of binder and super disintegrating agents

Total 9 batches were prepared for optimization of binder and disintegrating agent quantities as mentioned in Table. 4.5. Sodium starch

glycolate (SSG) and Povidone K-30 (K-30) selected as super disintegrating agent and binding agent, respectively. Binder is responsible for making of coherent mass where as disintegrate is responsible for breaking of naplet up on contact of dissolution medium. Lactose monohydrate (LMH) was used as a diluents in preparation of Naplet.

4.2.4.3. Sub and enteric coating of naplet

The dried Naplet were subjected to sub coat (2 % w/w) and enteric coating (4 % w/w) by spray coating technique using conventional coating pan (Macro Scientific Works, India). The sub coating was used for making of inert core of Naplet and improving of coating elegance. The solutions of Eudragit EPO and L-100 were used for sub and enteric coating as mentioned in Table 4.6. Coating process was carried out with overnight stirred coating solutions at bed temperature below 30±2 °C and weight gain was periodically checked till it reached to the weight of sub (2%) and enteric (4%) coat. The coated naplets were air dried overnight and finally vacuum-dried (Decibel Digital Technology, India) at temperature and pressure conditions 35 °C and 400 mm Hg, respectively for 2 h.

Table. 4.4. List of excipients and quantities used in preparation of Naplet

Formula	Quantity(mg) per naplet	Percentage (%) per naplet	Category
BBR –NP	6.25 (≈ 3 mg BBR)	25	Active ingredient
LMH	16.50 - 18.25	66 - 73	Diluent
K-30	0.25 – 1.00	1-4	Binder
SSG	0.25 - 1.25	1-5	Super disintegrate
Water	q.s	q.s	Preparation vehicle
Total	**25**	**100**	

Table. 4.5. Composition of different batches of naplets

Batch	Ingredients (mg)			
	BBR-NP	**LMH**	**SSG**	**K-30**
1	6.25	18.25	0.25	0.25
2	6.25	18.00	0.25	0.50
3	6.25	17.50	0.25	1.00
4	6.25	17.75	0.75	0.25
5	6.25	17.50	0.75	0.50
6	6.25	17.00	0.75	1.00
7	6.25	17.25	1.25	0.25
8	6.25	17.00	1.25	0.50
9	6.25	16.50	1.25	1.00

BBR-NP: Berberine nanoparticle, LMH: Lactose monohydrate, SSG: Sodium Starch Glycolate, K-30: Povidone. Water as q.s and total weight of the naplet was 25 mg.

Table. 4.6. Excipients and quantities used for sub and enteric coating of naplets

Excipients	Quantity (mg)	Percentage (%)	Category
Eudragit EPO	0.5	2	Sub coating
Eugragit L-100	1	4	Enteric coating
Talc	0.5	2	Anti adherent

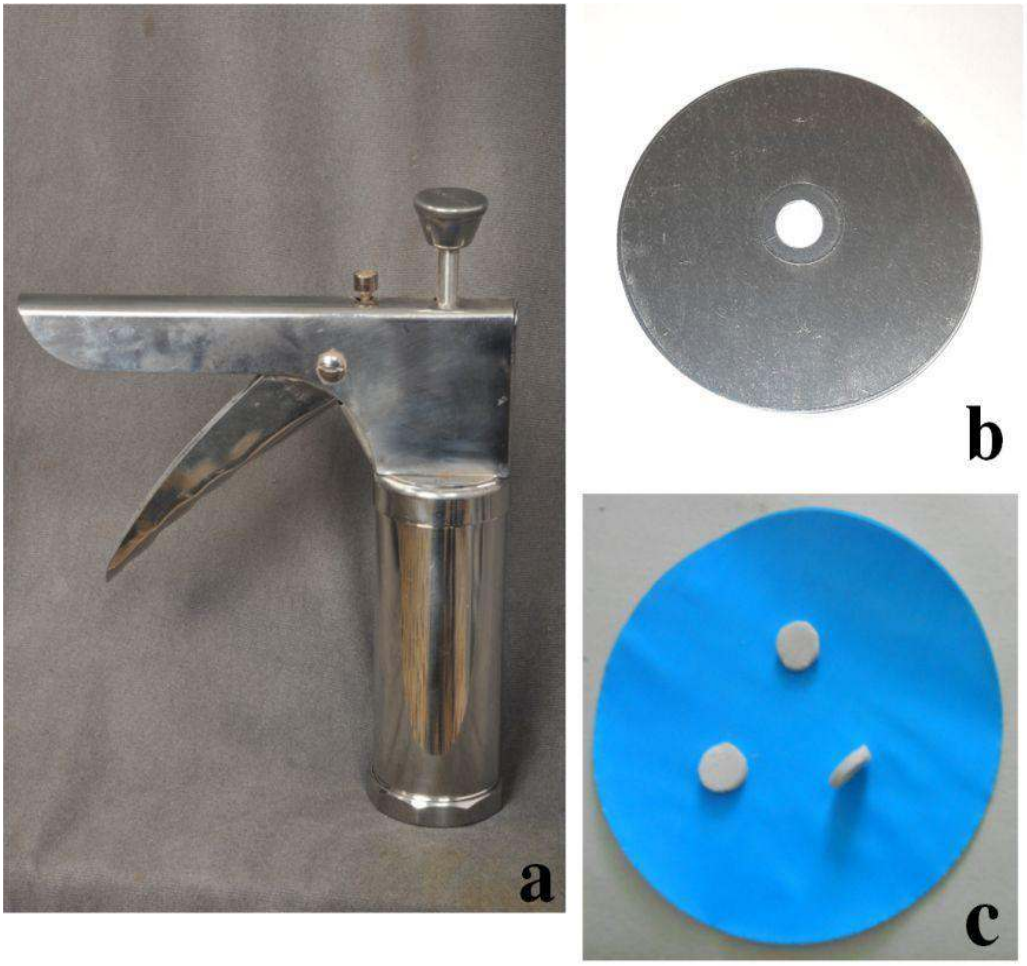

Figure. 4.2. (a) Stainless steel naplet device (b) Naplet disc and (c) Uncoated naplet

4.2.5. Characterization of Nanoparticles and Naplet

4.2.5.1. Particle size, polydispersity index and zeta potential

The particle size (PS), polydispersity index (PDI) and zeta potential (ZP) were estimated by DelsaTM Nano C (Beckmann Coulter, USA). The PS and PDI were measured by dynamic light scattering technique at 25 °C. Samples were scattered at an angle of 165°. Data were fitted by the method of inverse "Laplace transformation" and CONTIN. Polydispersity index indicate the distribution of particle size of nanoparticles. The ZP of nanoparticles was measured from the electrophoretic mobility under electric field using Helmholtz – Smoluchowski equation.

4.2.5.2. Entrapment efficiency

The entrapment efficiency (EE) was estimated with method described by Bisht et al (Bisht *et al.,* 2007).. The nanodispersion around 500 µl was placed in the upper chamber of Nanosep® centrifuge tubes containing ultrafilter with molecular weight cut-off 100KD (Pall Life Sciences, India). Nanosep® was centrifuged at 5000 rpm for 30 minutes using cooling centrifuge (Remi, C-24, India) at 4°C. The filtrate was collected from lower chamber and unentrapped BBR was estimated by HPLC method. The EE was calculated by the following equation:

$$EE\ (\%) = [(At - Aun)/ At] \times 100$$

where At and Aun denotes the amount of total drug and analyzed amount of un-entrapped drug in the nanodispersion, respectively.

4.2.5.3. Fourier Transform – Infra Red (FT-IR)

Fourier Transform – Infra Red (FT-IR) spectrums were obtained on a FT-IR spectrometer by the conventional KBr pellet method. The samples were grounded gently with anhydrous KBr and compressed to form pellet. The scanning and the resolution range were 400 – 4000 cm^{-1} and 4 cm^{-1}, respectively.

4.2.5.4. Differential Scanning Calorimeter (DSC)

Thermograms of each sample were recorded by differential scanning calorimeter (TA Instruments Q 1000, Delaware, USA) equipped with intracooler and refrigerated cooling system. Each sample was placed in an aluminum pan and hermetically sealed with an aluminium lid. All measurements were performed at 5 °C/min heating rate and nitrogen was purged at 50 ml/min through cooling unit.

4.2.5.5. Powder X-Ray Diffraction (PXRD)

Powder X-ray diffraction patterns were traced employing X-ray diffractometer (Model No. 3000, Seifert) for the samples, using Ni-filtered Cu–K radiation, a voltage of 40 kV, a current of 30 mA radiation scattered in the crystalline regions of the sample, which was measured with a vertical goniometer. Patterns were obtained by using a step width of 0.04° with a detector resolution in 2q (diffraction angle) between 10° and 80° at ambient temperature.

4.2.5.6. Transmission Electron Microscope (TEM)

The morphology of the samples was observed using transmission electron microscope (TEM) with JEM – 100S TEM (Philips Morgagni, 268). The samples were placed on a carbon coated copper grid to leave a thin film on the grid and negatively stained with 1% phosphotungustic acid. The sample grid was allowed to dry thoroughly at room temperature and was viewed with appropriate magnification.

4.2.5.7. Scanning Electron Microscope (SEM)

The surface morphology of the nanoparticles in dispersion and naplet were determined by a high resolution Field Emission Scanning Electron Microscopy (SEM, FESEM-Carl Zeiss, Supra 40) operated at an accelerating voltage 3.0 kV. Prior to imaging, samples were sputter coated for 50 s with platinum using a JEOL JFC-1200 fine coater to make the conducting specimens.

4.2.5.8. Disintegration of Naplet

The standard USP test (USP 27) with discs was employed to assess the disintegration time of uncoated naplet, using an Erweka disintegration tester (model ZT31, Germany). Tests were carried out with uncoated naplet in 800 mL of distilled water at 37 ± 0.5 °C. The disintegration time was defined as the time necessary for complete disintegration of the naplets. All tests were run in triplicate.

4.2.5.9. Redispersion of Naplet

The redispersion of nanoparticle was measured by indirect method i.e measurement of drug equivalent to nanoparticles. The naplet equivalent to 3 mg of BBR nanoparticle was placed in a conical flask with 25 ml of phosphate buffer (pH 6.8) and kept shaking for 10 min at 60 rpm. The obtained disintegrated solution was passed through whatmann filter paper and filtrate (nanoparticle dispersion) was measured for drug content. Nanoparticle dispersion (1 ml) was dissolved in 1:1 mixture of acetone and ethanol (9 ml) and was filtered through 0.45 µ syringe filter (Whatman). The filtrate sample was measured by HPLC method. Each sample was analyzed in triplicate. The total drug content (TDC) was calculated by using the equation:

TDC (%) = concentration × dilution factor × volume of formulation / Drug amount used × 100.

4.2.5.10. Hardness and Friability of Naplet

The naplets crushing strength was determined using hardness tester (type TBH 28, Erweka, Germany) and the friability was determined using Roche friability tester (Erweka, Germany). The drum containing pre-weighed naplets were rotated at 25 rpm for 4 min and the naplets were removed, undusted and accurately weighed. Friability was calculated by using following formula:

F (%) = (W0-Wf/ W0) X 100

Where W0 is the initial weight of naplets and Wf is the weight after friability test

4.2.6. *In-vitro* Drug Release and Kinetic Study

4.2.6.1. BBR-NP and BBR-SCNP

The drug release study was performed in pH 7.4 saline phosphate buffer (PBS) using the dialysis bag method. The dialysis membranes (thickness 0.025 mm, mol. wt. cut off 12000–14000 Da) were soaked overnight in the dissolution media before study to ensure thorough wetting of the membrane. Both type of nanoparticle containing BBR (3 mg equivalent) along with 2 ml of the PBS was placed into the dialysis bag, two ends were tied and fixed by clamps. The dialysis bag was submerged into a beaker containing 75 ml of medium kept at 37 ± 0.5°C and stirred magnetically at 100 rpm. Samples were withdrawn at preset intervals with immediate replacement of equal volumes of the fresh medium to maintain sink condition. The samples were filtered through 0.22 μm syringe filters and the BBR content was determined by HPLC method. A similar study was also conducted with pure BBR. All the studies were carried out in triplicate (n=3).

4.2.6.2. Naplet

The drug release study was performed in pH 1.2 (acidic) and pH 6.8 (phosphate) media using the dialysis tube method. The dialysis membranes (thickness 0.025 mm, mol. wt. cut off 12000–14000 Da) were

soaked overnight in the dissolution media before study to ensure thorough wetting of the membrane. BBR-NP dispersion and naplet (equivalent to 3 mg of BBR) along with 2 ml of the PBS was placed into the dialysis tube and mounted tightly with experimental thread. The dialysis tube was submerged into a glass beaker containing pH 1.2 medium for initial three hours followed by pH 6.8 medium in 300 ml for 24 h at 37 ± 0.5°C and stirred magnetically at 100 rpm. Samples were withdrawn at preset intervals with immediate replacement of equal volumes of the fresh medium to maintain sink condition. The samples were filtered through 0.45 µm syringe filters and the BBR content was determined by HPLC method. All the studies were carried out in triplicate (n=3).

4.2.7. Stability Studies

4.2.7.1. Study at storage conditions (shelf life)

BBR-NP and naplets were stored at 25 ± 2 °C/60 ± 5% RH (Labtop stability chamber, Skylab Instrument and Engineering Pvt. Ltd., India) where as BBR-SCNP stored at cool condition (8 ± 2 °C) for 180 days and characterized for PS, PDI, ZP (dispersed in water) during storage period.

4.2.7.2. Study in simulated biological fluids

BBR-NP and BBR-SCNP (equivalent 10 mg of drug) were primarily dispersed in 1 ml of water separately to made nanoparticle dispersion and incubated in 9 ml of simulated biological fluids of pH 7.4 and 6.8 with

or without 20 mM of sodium taurocholate and human plasma for 30 min at 37°C.

4.2.8. Pharmacokinetic Study

4.2.8.1. HPLC bio-analytical method development

The bio-analytical method described by Kheir et al was used for quantification of BBR in plasma samples (Kheir *et al.*, 2010). Bio-analytical method was developed and validated with reverse phase HPLC system consisting of binary pumps (Waters Corp., Milford, MA, USA), a diode array detector (Waters 2998) and operating software (Empower Node 2054). A rheodyne manual injector (USA) attached with 20 µl sample loop was used for loading the sample. A C18 reverse-phase ODS2 column (250 × 4.6 mm 5 µm) (Waters Corp., Milford, MA, USA) and a C18 guard column (4.6 × 10 mm) were utilized for BBR separation and quantification. The mobile phase was water (0.2% triethylamine, pH 3.0 by phosphoric acid)–methanol (72:28, v/v), filtered through a 0.45 µ Millipore filter and degassed prior to use.

The flow rate was 1.0 ml/min and the column was maintained at a constant temperature (25 ± 1 °C). Drug detection was performed at a wavelength of 347 nm (λ max). Plasma standard curves were constructed in the range of 0.1 – 1 µg/ml (triplicate) and validated for linearity, precision, accuracy, limit of quantification (LOQ) and limit of detection (LOD).

4.2.8.2. Oral single dose animal study

The animal experimental protocol was approved by the Animal Ethical Committee of the Institute of Medical Sciences, Banaras Hindu University, Varanasi, India ((No. Dean/10-11/101). Healthy, four weeks either sex old Wistar rats (body weight 250 ± 20 gms) were used for this study. Rats were housed in polypropylene cages with bedding of rice husk. The rats were acclimatized prior dosing of seven days in laboratory conditions of 25±2°C with light/dark cycles of each of 12 h. They were freely accessed with standard pellet diet (Hindustan Uniliver, India) and water *ad libitum*. Single dose pharmacokinetic study was designed with five groups comprising six animals in each group (n = 6) as depicted in Table 4.7. Rats were fasted before the 12 h of experiment and allowed free access of water during the study period. Dose was calculated for each animal in group as per animal body weight and administered via oral route. First and fifth group received aqueous solution of BBR (100 mg/kg body weight). Aqueous suspension of verapamil in 0.3 % w/v of CMC (25 mg/kg body weight) administered 60 min prior to dosing of BBR in animals of fifth group. Second, third and fourth group received macro (physical mixture of BBR, PCL and vitamin E TPGS), BBR-NP and BBR-SCNP (equivalent to 100 mg/kg body weight of BBR). Blood samples (approx. 0.3 ml) from the retro orbital plexus of rats were collected in heparin coated micro centrifuge tubes at pre fixed time points (0, 0.25,

0.50, 1, 1.5, 2, 3, 6, 9, 12 and 24 h) from the rats of respective groups. Plasma were collected from blood samples by centrifugation (5000 rpm for 10 min at 4°C) and stored at -40°C until analyzed. Liquid – liquid extraction method was employed for extraction of BBR from the plasma sample. Briefly, 100 µl of plasma with 1 ml of methanol was placed in 2 ml of micro centrifuge tube and the mixture was vortex-mixed for 30 s and centrifuged at 12000 rpm for 10 min. Then, the supernatant was collected into clean centrifuge tube, placed in a water bath and evaporated to dryness under a stream of nitrogen at 40°C. The residues were reconstituted in 100 µl methanol and vortex mixed for 30 s. Finally, 20 µl aliquot of the supernatant was injected into the HPLC system for quantification of BBR.

Pharmacokinetic parameters, such as peak plasma concentration (C_{max}), time to reach peak concentration (T_{max}), area under the curve from time zero to last measured concentration (AUC), the time span during which the plasma concentrations were at least 50% of the C_{max} value ($t_{1/2}$) and clearance (Cl) were obtained for each group by non-compartmental pharmacokinetic models using WinNonlin® (5.1 version) software.

Table 4.7. Animal groups used in pharmacokinetic study

Group	Dose of animals
I	BBR pure solution
II	BBR macro formulation (Physical mixture of BBR, PCL and vitamin E TPGS)
III	BBR-NP
IV	BBR-SCNP
V	BBR pure solution + Verapamil in 0.3 % CMC
VI	Control treated with water

4.2.9. Haemocompatibility Study

4.2.9.1. Haemolysis assay

Haemolysis experiments were performed according to the method of Bender et al (Bender *et al.*, 2012). Human blood samples used were freshly obtained from authorized blood bank (Andhra Blood Bank, A.P). Initially, 5 ml blood was centrifuged at 1600 rpm for 5 min. Supernatant plasma surface layer was removed and sediment RBC pellet was separated and washed thoroughly with normal saline solution. The RBC pellet was diluted with 25 mL of normal saline solution. Pure BBR, BBR-NP and BBR-SC NP (equivalent to 10 µg and 100 µg of BBR) were added in 2 ml of RBC suspension separately. Similarly, BBR-NP and BBR-SCNP placebos (i.e. equivalent weight to BBR-NP and BBR-SCNP) were also added to RBC suspension. Positive (100 % lysis) and negative (0% lysis) control samples were prepared by adding equal volumes of Triton X-100 and normal saline, respectively to RBC suspension. The samples were

incubated at 37°C for 3h. The samples were slightly shaken once for every 30 min to re-suspend the RBCs and NP. After 3 h, the samples were centrifuged at 1600 rpm for 5 min and 100 µL of supernatants were incubated for 30 min at room temperature to allow haemoglobin oxidation. Oxyhemoglobin absorbance was measured spectrophotometrically at 540 nm. Haemolysis percentages of the RBC were calculated using the following formula (Bender *et al.,* 2012):

% Haemolysis = (absorbance of sample - absorbance of negative control)/ (absorbance of positive control - absorbance of negative control)

4.2.9.2. LDH assay

The lactate dehydrogenase (LDH) enzyme release from RBC was assessed spectrophotometrically using the LDH commercial kit (Span Diagnostics, India). All the samples were incubated in RBC suspension for 3 h as described in haemolysis assay method. After that, the samples were centrifuged at 1600 rpm for 5 min and LDH released in supernatant was measured spectrophotometrically at 500 nm (U-1800, Hitachi). Equal volume of Triton X-100 was added to RBC suspension and treated as control. The concentration of LDH released was calculated using the following formula (Bender *et al.,* 2012).

$$\text{Lactate dehydrogenase (U L}^{-1}) = \frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{control}}}{\text{Abs}_{\text{standard}}} \times 150$$

Where Abs $_{sample}$ is the absorbance of the supernatant of the samples, Abs $_{control}$ is the absorbance of the supernatant of the RBC suspension added to the substrate reaction. Abs $_{standard}$ is the absorbance of supernatant of RBC suspension with LDH standard (150 U L^{-1}, according to the manufacturer specifications). All samples were analyzed in triplicate (n = 3).

4.2.9.3. Platelet aggregation

Platelet aggregation analysis was performed as described in method of Bender *et al*. Peripheral blood smears were prepared after the incubation of whole blood with NP to observe platelet changes due to the particle interaction. In brief, the samples were incubated with whole blood for 3 h at 37°C as mentioned in haemolysis and LDH assay. After incubation, peripheral blood smears were stained with Leishman's stain for 5-6 min (Span Diagnostics, India). Then, smears were rinsed by water and allowed to dry for few minutes. Dried smears were analyzed by optical microscope in immersion objective and images were captured using the digital system (Nikon Trinocular microscopic unit, model E-200, Japan).

The quantitative evaluation of platelet aggregation was carried using haematological counter (ABX Micros 60, Horiba-ABX, India) for all the samples before and after incubation. All samples were analyzed in triplicate (n = 3).

4.2.10. Toxicity study

The toxicity study was conducted as per OECD guidelines (section 4; Health Effects, 28-Day Oral Toxicity in Rodents). All animal experiments were conducted as per the approval of the Institutional Animal Ethical Committee, Banaras Hindu University, Varanasi, India. Healthy, four weeks old either sex Wistar rats (body weight 200 ± 50 gms) were used for this study. Rats were housed in polypropylene cages with bedding of rice husk. The rats were acclimatized prior dosing of seven days in laboratory conditions of 25±2°C with light/dark cycles of each of 12 h. The repeated oral dose toxicity study was designed with four groups comprising of six animals in each group (n= 6) as depicted in Table 4.8. Animals were given standard pellet diet (Hindustan Uniliver, India) and water *ad libitum* during the study period. Dose was calculated for each animal in group as per animal body weight and administered via oral route for 28 days. The first group received vehicle (water) considered as control, second group received aqueous solution of BBR (100 mg/kg body weight), third group received BBR-SCNP (equivalent to 100 mg/kg body weight of BBR) and fourth group received placebo nanoformulation (100 mg/kg body weight).

The rats were observed daily in study period of 28 days for different parameters such as animal appearance, food and water intake, urination, defecation, fecal matter color and mortality.

4.2.10.1. Histopathological study

On the 29th day of study (post day of experiment), three animals from each group were sacrificed by cervical dislocation (after withdrawn of blood). Organs such as liver, kidney and small intestine were isolated out from each animal. Separated organs were rinsed thoroughly with normal saline solution and removed unwanted tissue matter. Each organ was weighed and dissected organ tissues were preserved separately in 10 % neutral formalin solution for microtomy. Later, these tissues were dehydrated and embedded in paraffin, sliced in 4-5 µm thickness and stained with haemotoxylin – eosin dye for anatomical microscopic observations.

4.2.10.2. Biochemical estimations

On the 29th day of study (post day of experiment period), randomly selected three animals from each group was anaesthetized with ether and blood was withdrawn by cardiac puncture. Collected blood was centrifuged (3500 rpm, 10 min at 4°C) and separated serum was stored at -20°C until biochemical estimations. Liver functional enzymes such as (AST), (ALT) and (ALP) and Kidney functional enzymes such as urea and creatinine levels were estimated using standard kits from Span Diagnostics, India.

Table 4.8. Animal groups used in toxicity study

Group	Dose of animals
I	Control: Aqueous Vehicle (Water)
II	BBR aqueous solution
III	BBR-SCNP
IV	Placebo-SCNP

4.3. Statistical analysis

The results were expressed as mean ± standard deviation. Statistical comparisons of the experimental results such as solubility, stability studies and biochemical estimations were performed by the Student's t-test, one and two way ANOVA (as applicable), respectively. In all cases, p-value less than 0.05 were considered to be significant. Post hoc analysis such as Tukey and Newman-Keuls were also applied based on suitability.