# Chapter 8

# *IN VIVO* STUDIES

## 8.1 Introduction

### 8.1.1 Acute toxicity study

*In vivo* acute toxicity study in an animal model is an important part in the process of drug and formulation development. Precise determination of Median lethal dose ($LD_{50}$) i.e. dose of drug required to kill the half of the treated population can be precisely determined by this study. The foremost purpose of $LD_{50}$ determination in formulation development is that it is a method where a comparative study can be performed between novel drug products with established ones. Several methods have been developed to minimize the number of experimental animals used for studying toxicity. Fixed dose procedure (FDP) and Up-down procedure (UDP) are the two methods which are used to get an insight into the toxicity of formulations or chemicals. Among these two methods UDP requires minimum number of experimental animals (6-10) and provide results in terms of $LD_{50}$. Whereas, FDP yields data which can be used for hazard classification system [1]. Thus, UDP procedure of organization for economic cooperation and development (OECD) was used to perform *in vivo* acute toxicity study [2].

### 8.1.2 *In vivo* Pharmacokinetic study

In vivo experiments are planned and performed to predict the toxicity, corrosivity, and other safety variables as well as the effectiveness/efficacy of novel formulations. An appropriate animal model helps in understanding the fate or behavior of the developed novel formulation in *in vivo* condition. Although newer *in vitro* techniques have helped in reducing the number animals required in *in vivo* study [3]. However, it is necessary to find the ADME (absorption, distribution, metabolism and elimination) parameters of the formulations and drugs before proceeding to clinical trials [4]. Correlation of *in vitro* data with *in vivo* data can effectively predict the formulation behavior. The pharmacological effect of a drug is directly related to its concentration required, which is dependent upon the drug concentration present in blood/plasma. Hence, a time dependent blood/plasma monitoring for drug concentration i.e. pharmacokinetic study can be useful in conducting clinical trials. Rodents are the preferred animal model for conducting preliminary pharmacokinetic study.

Pharmacokinetic studies can be carried out by two methods i.e. non-compartmental and compartmental modelling. Non-compartmental methods assess the drug exposure by estimation of area under curve (AUC) by plotting concentration vs. time graph. Whereas, compartmental models assess the kinetic models by plotting concentration vs. time graph. Non-compartmental models are considered versatile as they do not follow any compartmental

specific model. Also, the obtained results are accurate and acceptable for bioequivalence studies. The results of the transformation that drugs or formulations undergoes in an animal and the factors that regulate this fate are interrelated.

### 8.1.3 *In vivo* Biodistribution study

The pharmacokinetic parameters of parent drug and drug encapsulated in nano carriers are often different. Hence, it is important to study the biodistribution patterns of novel formulations. Non-specific biodistribution of cytotoxic drugs can cause serious side effects, to counter this issue encapsulation has been proven advantageous. However, nano-sized carriers impose a threat of bioaccumulation to the sites/organs other than the site of action of drug which can cause unwanted side effects. Also, physical properties like size, surface charge and surface chemistry of the carrier system plays a pivotal role in biodistribution [5]. Quantification of drug concentration by sophisticated analytical techniques in highly perfused organs provides an insight into the biodistribution of drugs and formulations.

*In vivo* Biodistribution study of novel formulations can also be performed by non-invasive technique like gamma (γ) scintigraphy. Radiolabelled drug and formulation bearing it when administered into the animal, upon a real time imaging provides the instantaneous data of biodistribution. Various radiotracers like Technetium-99m ($^{99m}$Tc), Indium-111, Gallium-67 are used in gamma scintigraphy [6]. Every radiotracer has different half-life, thus, study protocol is taken into consideration in choosing the radiotracer. Gamma camera is employed to take the scintigraphs. Total gamma counts in relation with site of interest counts are used to study the biodistribution throughout the animal.

## 8.2 Materials and Equipments

### 8.2.1 Materials

Gemcitabine Hydrochloride (GCH) was obtained as a gift sample from Sun Pharmaceutical Industries Ltd., Vadodara, India. Vinorelbine tartrate (VLB) was obtained as a gift sample from Cipla Ltd. Mumbai, India. Soyabean Phosphatidylcholine (SPC) 95% (PhospholiponVR 90 G) and 1,2- distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino (polyethylene glycol)-2000] (DSPE-PEG 2000) were obtained as gift sample from Lipoid GmbH (Ludwigshafen, Germany). Cholesterol, Mannitol, Potassium oleate, Stannous chloride (SnCl2.2H2O) and Diethylenetriaminepentaacetic acid (DTPA) along with HPLC grade Methanol, Formic acid, Acetonitrile, Ammonium acetate were purchased from Sigma Aldrich (St. Louis, MO, USA). Sodium chloride, Potassium chloride, Disodium phosphate and Monopotassium phosphate were purchased from Loba Chemie, Mumbai, India. For LCMS analysis Direct-Q® Water

Purification System, Millipore (Bedford, MA, USA) was used throughout the study. Membrane filter (0.22 microns) was purchased from Pall Life sciences, USA. Silica gel (SG)-coated fiberglass sheet was obtained from Gelman Sciences Inc., Ann Arbor, MI. Sodium pertechnetate separated from Molybdenum-99 by solvent extraction method was procured from the Regional Centre for Radiopharmaceutical Division (Northern Region), Board of Radiation and Isotope Technology, Delhi, India. All other chemicals used were obtained from authentic source and were of Analytical Reagent grade.

### 8.2.2 Equipments

- Analytical Weighing Balance (ATX 224, Shimadzu, Japan)
- Rotary evaporator (IKA RV10, Karnataka, India)
- IKA®-WERKE Eurostar power control-visc (IKA, Bengaluru, India)
- Ultrasonic Bath 120W (Vibronics Co. Pvt. Ltd., Mumbai, India)
- pH meter (PICO pH meter, LABINDIA Analytical, Mumbai, India)
- Cooling centrifuge (Remi Equipments, Mumbai, India)
- Vortex Mixer (Spinix-Vortex Shaker, Tarsons, India)
- LCMS-MS (ekspertTM ultraLC with ekspertTM ultraLC 100 pump system (eksigent-AB Sciex, Framingham, MA) coupled with 3200 QTRAP mass spectrometer (AB Sciex, Framingham, MA)
- Gamma Counter (Caprac T well counter, Capintec NJ, United States)
- Gamma Camera (Single Photon Emission Computerized Tomography (SPECT, LC 75–005, Diacam, Siemens AG, Earlangan, Germany)

## 8.3 Methods

### 8.3.1 Institutional Animals Ethics Committee (IAEC) approval

Prior approval for conducting all *in vivo* animal experiments was obtained from Institutional Animals Ethics Committee (IAEC) of the Pharmacy Department, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India (Protocol No. MSU/IAEC/2014-15/1409). All animal experiments protocols were in accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India, New Delhi, India.

### 8.3.2 Hemocompatibility Study

Hemocompatibility Study was performed to ensure the compatibility between formulation dispersion and red blood cells (RBCs). Briefly, freshly collected rat blood from retro orbital

plexus was taken in 2 ml of heparinized eppendorf tubes, followed by centrifugation at 3000 rpm for 10 minutes at 4 °C. The RBCs pellet was collected and resuspended into normal saline (0.9 %w/v sodium chloride in water) and centrifuged, this procedure was repeated thrice to remove any plasma component. 1 ml of RBCs suspended in normal saline was taken and separately 1 ml of test substances i.e. Triton x-100 (1% v/v, +ve control), 0.9 %w/v normal saline (-ve control), Standard GCH solution, GCH loaded Spherulites, Standard VLB solution and VLB loaded Spherulites were added. The resulting dispersions were incubated for 1 hour at 37 °C. RBCs were observed under microscope for any aggregation or rupture [7].

### 8.3.3 Acute Toxicity Study

Acute toxicity study was performed for determining the median lethal dose ($LD_{50}$). Female Swiss Albino Mice (8-12 weeks old) weighing 25-30 gm were randomly allocated to the groups as Standard GCH solution, GCH loaded Spherulites, Standard VLB solution and VLB loaded Spherulites (n=6). All the animals were kept on fasting condition for 3-4 hours prior to dosing but allowing free access to water.

All the animals were dosed (injected via tail vein) in sequence usually at 48 hours interval. Selected dosing range was 100, 200, 300, 400 and 500 mg/kg. Animals were observed closely for the sign of toxicity in terms of decrease in weight, convulsions, rashes, akinesia. If the animal survived during first 48 hours interval, second animal was dosed at a higher dosing level. Dosing at higher dose was stopped if the signs of toxicity during initial 48 hours do not provide the chances of survival of the animal. Dosing was continued depending on the fixed time interval of 48 hours and outcomes were recorded of all animals up to 14 days in terms of death. Close observation of animals was done every 30 minutes for at least 4 hours post injection and three times a day thereafter [8,9].

### 8.3.4 *In vivo* Pharmacokinetic study

Female Sprague-Dawley (SD) rats of 6-8 weeks of age, weighing 220±20 g were used as experimental animal model for pharmacokinetics studies. Animals were kept in polypropylene cages (38cm × 23cm × 10cm) under controlled laboratory conditions (Temperature 25±2 °C; Humidity 50±20 %). Alternate 12 hours light and dark cycle was maintained. 3 rats per cage were housed in animal house and were fed ad libitum with animal feed allowing free access to drinking water.

Pharmacokinetic study was performed to establish the relationship between drug dose and its plasma concentration with respect to time. About 1 mL of Standard GCH solution, GCH loaded non-PEGylated Spherulites and GCH loaded PEGylated Spherulites, each containing 0.5 mg

of GCH, was administered through tail vein of rat (n=6). Similarly, About 1 mL of Standard VLB solution, VLB loaded non-PEGylated Spherulites and VLB loaded PEGylated Spherulites, each containing 0.5 mg of VLB, was administered through tail vein of rat (n=6). 0.5 mL of blood sample was withdrawn from retro-orbital plexus at the predetermined time points of 0.25, 0.5, 1, 2, 4, 8, 12 and 24 hours. 0.05 mL of Heparin (100 IU) was added in the tube before blood collection to prevent the sample from coagulation. Plasma was separated from blood sample by centrifuging (Remi Equipments, Mumbai, India) the eppendorf tube for 5 minutes at 2200 ×g at 4 ºC. Separated plasma was obtained as supernatant. It was collected and stored at -20 ºC till further analysis. All the pharmacokinetic parameters like concentration ($C_{max}$), time to attain maximum concentration ($T_{max}$), Area under curve (AUC), Half-life ($t_{1/2}$), Volume of Distribution (Vd), Mean residence time (MRT) and rate of clearance (Cl) were calculated after analyzing the samples by LCMS-MS method. Thermo Kinetica Version 5.0 (Build 5.0.11) (Thermo Fisher Scientific, Waltham, MA, USA) was used to calculate the pharmacokinetic parameters.

Two way ANOVA followed by Bonferroni Post-tests was performed in order to reveal the statistical significance in pharmacokinetic parameters. Statistical analysis was done by using GraphPad Prism Version 5.00 for Windows (GraphPad Software, La Jolla, California, USA).

### 8.3.5 *In vivo* Biodistribution study by LCMS-MS analysis

Female SD rats of 6-8 weeks of age, weighing 220±20 g were used as experimental animal model for pharmacokinetics studies. Animals were kept in polypropylene cages (38cm × 23cm × 10cm) under controlled laboratory conditions (Temperature 25±2 °C; Humidity 50±20 %). Alternate 12 hours light and dark cycle was maintained. 3 rats per cage were housed in animal house and were fed ad libitum with animal feed allowing free access to drinking water.

*In vivo* biodistribution study was performed to study the distribution pattern of the plain drug and the formulations. Briefly, About 0.6 mL of Standard GCH solution, GCH loaded non-PEGylated Spherulites and GCH loaded PEGylated Spherulites, each containing 0.3 mg of GCH, was administered through tail vein of rat (n=3). Similarly, About 0.6 mL of Standard VLB solution, VLB loaded non-PEGylated Spherulites and VLB loaded PEGylated Spherulites, each containing 0.3 mg of VLB, was administered through tail vein of rat (n=3). At Predetermined time points the animals administered with test sample were euthanized and highly perfused organs like Heart, Lungs, Liver, Spleen and Kidneys were isolated. Isolated organs were blotted with absorbent paper and immediately homogenized using organ homogenizer, followed by centrifugation for 5 minutes at 2200 ×g at 4 ºC. Supernatant was

collected and stored at -20 ºC till further analysis. The drug concentration present in the isolated organs was estimated using LCMS-MS method.

### 8.3.6 *In vivo* Biodistribution study of GCH and GCH loaded formulations by Gamma (γ) scintigraphy [10]

#### 8.3.6.1 Radiolabelling of GCH with Technetium-99m ($^{99m}$Tc)

Briefly, 2 mg GCH was dissolved in 1 ml Phosphate Buffer Saline (PBS) pH 7.4 followed by the addition of 75 µg of $SnCl_2.2H_2O$. The pH of the solution was adjusted to 6.5. The contents were filtered through 0.22 µm membrane filter (Millipore Corporation, Bedford, MA USA). Freshly prepared $^{99m}TcO^-_4$ (74 Mbq) was added to the contents, mixed and incubated for 20 minutes at room temperature.

#### 8.3.6.2 Quality Control of $^{99m}$Tc-labelled GCH

$^{99m}$Tc-labelled GCH was checked for labelling efficiency by Instant Thin Layer Chromatography (ITLC) using 100% Acetone as mobile phase. Strips (1*12 cm) of silica gel (SG)-coated fiberglass sheets (Gelman Sciences Inc, Ann Arbor, MI) were taken. 2-3 µL of radio-labelled complex was applied at 1 cm above the bottom of ITLC strip with the help of glass capillary tube. The strip was kept in the glass chamber containing mobile phase and allowed to develop till the mobile phase reached at 10 cm from the point of sample application. The strip was then cut into 60:40 (Bottom: Top) proportion and the radioactivity counts were determined in each portion by well-type gamma counter (Caprac T well counter, Capintec NJ, United States). The counts of free $^{99m}$Tc-pertechnetate were determined which moved along with the mobile phase (Rf=0.9). $^{99m}$Tc-labelled GCH along with reduced/hydrolyzed technetium remained at the point of sample application on the ITLC strip. The colloid formation was determined in pyridine: acetic acid: water (3:5:1.5 v/v) as mobile phase. The % labelling efficiency of $^{99m}$Tc-GCH was calculated by subtracting % activity moved with the solvent front using pyridine: acetic acid: water as a mixture from % activity moved with the solvent front using acetone.

#### 8.3.6.3 Radio-complex stability

The stability study of $^{99m}$Tc-GCH was studied in human serum and normal saline. 200 µl of radiolabelled complex was added to 500 µl of human serum and normal saline and incubated for 30 min at 37°C. The labelling efficiency was determined at regular time intervals by ITLC with 100% acetone as mobile phase.

### 8.3.6.4 Transchelation Study (DTPA Challenge)

2.5, 5 and 7.5 mM solution of DTPA was prepared in distilled water. 500 µl of $^{99m}$Tc-GCH was incubated for 2 hours at 37°C with different strength viz. 2.5, 5 and 7.5 mM of DTPA solution. The effect of incubation with DTPA on the radiolabelled complex was observed by ITLC using 100% acetone as mobile phase. The developed ITLC strip was then cut into 3 parts (0-5, 5-8, 8-10 cm) and radioactivity counts were determined in well-type gamma counter (Caprac T well counter, Capintec NJ, United States).

### 8.3.6.5 Biodistribution studies in Rats

All animal experiments conducted were approved by the Institutional Animals Ethics Committee of the Institute of Nuclear Medicine and Allied Sciences (INMAS), DRDO, New Delhi, India. Experimental animals used were healthy female SD rats weighing 200±4 g (14 weeks), kept on normal diet with free access to water. Animals were divided into 3 groups, $^{99m}$Tc-GCH loaded non-PEGylated spherulites, $^{99m}$Tc-GCH loaded PEGylated spherulites and standard $^{99m}$Tc-GCH solution.

$^{99m}$Tc-GCH loaded PEGylated and non-PEGylated spherulites were prepared by procedure mentioned in preparation of Spherulites section except the overnight hydration step. Dose of the drug to be administered in rat was calculated using the formula:

$$Human\ equivalent\ dose\ \left(\frac{mg}{kg}\right)(HED) = Animal\ dose\ \left(\frac{mg}{kg}\right) * \frac{Animalkm}{human\ km} \dots\dots\dots\dots (1)$$

where, Km is body weight (kg) divided by body surface area ($m^2$); the factor that varies for all animals.

0.6 ml of $^{99m}$Tc-GCH (1.48 MBq) loaded PEGylated and non-PEGylated spherulites and standard drug solution, each containing 0.3 mg of GCH, was injected in tail vein of rat. Rats were anaesthetized by injecting ketamine (100 mg/kg) through intraperitoneal route and were fixed on a board. Imaging was carried out at predetermined time points viz. 0.25, 0.5, 1, 2, 3, 4 hours using a Single Photon Emission Computerized Tomography (SPECT, LC 75–005, Diacam, Siemens AG, Earlangan, Germany) gamma camera. Deposition of intravenously administered formulation in whole body was calculated by taking into account gamma camera attenuation, background, and radioactive decay. Lung accumulation of the injected dose was expressed as a percentage of the whole-body deposition.

Two way ANOVA followed by Bonferroni Post-tests was performed in order to reveal the statistical significance. Statistical analysis was done by using GraphPad Prism Version 5.00 for Windows (GraphPad Software, La Jolla, California, USA).

### 8.3.7 *In vivo* Biodistribution study of VLB and VLB loaded formulations by Gamma (γ) scintigraphy [11]

#### 8.3.7.1 Radiolabelling of VLB with $^{99m}$Tc

Briefly, 2 mg VLB was dissolved in 1 ml double distilled water and 50µg of $SnCl_2.2H_2O$ was added to this solution. The pH of the solution was adjusted to 6.5-7.0 using 0.5M Sodium bicarbonate. The resulting solution was passed through 0.22 µm membrane filter (Millipore Corporation, Bedford, MA USA). Freshly prepared $^{99m}TcO^-_4$ (74 Mbq) was added to the contents, mixed and incubated for 20 minutes at room temperature.

#### 8.3.7.2 Quality Control of $^{99m}$Tc-labelled VLB

Labelling efficiency of $^{99m}$Tc-labelled VLB was checked by Instant Thin Layer Chromatography (ITLC) using Silica gel (SG)-coated fiberglass sheets (Gelman Sciences Inc, Ann Arbor, MI) and were cut into strips (1*12 cm). 100% Acetone was used as mobile phase. 2-3 µL of radio-labelled complex was applied by glass capillary tube at 1 cm above the bottom of ITLC strip. A glass chamber saturated with mobile phase was taken and the strip was kept inside. Strip was allowed to develop at a distance of 10 cm from the point of sample application.60:40 (Bottom: Top) proportion of the strip was cut and kept in the well-type gamma counter (Caprac T well counter, Capintec NJ, United States) to measure the radioactivity counts. Free $^{99m}$Tc-pertechnetate moves along with the mobile phase (Rf=0.9). Reduced/hydrolyzed technetium along with $^{99m}$Tc-labelled VLB remained at the bottom of ITLC strip where the sample was applied. The colloid formation was determined in pyridine: acetic acid: water (3:5:1.5 v/v) as mobile phase. The % labelling efficiency of $^{99m}$Tc-VLB was calculated by subtracting % activity moved with the solvent front using pyridine: acetic acid: water as a mixture from % activity moved with the solvent front using acetone.

#### 8.3.7.3 Radio-complex stability

Normal saline and human serum was used to evaluate the stability of $^{99m}$Tc-VLB complex. Radiolabelled complex (200 µl) was added to human serum and normal saline (500 µl) separately. The mixture was allowed to incubate for 30 min at 37°C. ITLC was carried out at regular intervals till 24 hours to determine the labelling efficiency using 100% acetone as mobile phase.

#### 8.3.7.4 Transchelation Study (DTPA Challenge)

DTPA solution was prepared in distilled water at concentration of 2.5, 5 and 7.5 mM. 500µl of $^{99m}$Tc-VLB was added to the different strength of DTPA solution and incubated for 2 hours at

37 ºC. ITLC of the radiolabelled complex was performed to know the effect of DTPA solution. 100% acetone was used as mobile phase. The developed ITLC strip was then cut into 3 parts (0-5, 5-8, 8-10 cm) and radioactivity counts were determined in well-type gamma counter (Caprac T well counter, Capintec NJ, United States).

**8.3.7.5 Biodistribution studies in Rats**

Healthy female SD rats weighing 200±4 g (14 weeks) were used for the in vivo experiments. Animals were divided into 3 different groups (n=3), $^{99m}$Tc-VLB loaded non-PEGylated spherulites, $^{99m}$Tc-VLB loaded PEGylated spherulites and plain $^{99m}$Tc-VLB solution. Prior approval from Institutional Animals Ethics Committee of the Institute of Nuclear Medicine and Allied Sciences (INMAS), DRDO, New Delhi, India was taken before commencing the animal experiments.

$^{99m}$Tc-VLB loaded PEGylated and non-PEGylated spherulites were prepared by procedure discussed earlier in the preparation section except overnight hydration step considering the half-life of $^{99m}$Tc as the radioactivity will get diminished. Animal dose to be administered was calculated using following formula:

$$Human\ equivalent\ dose\ \left(\frac{mg}{kg}\right)(HED) = Animal\ dose\ \left(\frac{mg}{kg}\right) * \frac{Animal km}{human\ km} \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

Where Km is body weight (kg) divided by body surface area ($m^2$); the factor that varies for all animals.

$^{99m}$Tc-VLB (1.48 MBq) loaded PEGylated and non-PEGylated spherulites and Plain drug solution in distilled water were injected in tail vein of rat. Injection volume was 0.6 ml each containing to 0.3 mg of VLB. Rats were anaesthetized by administering ketamine (100 mg/kg) intraperitoneal injection and were fixed on a board. Gamma Scintigraphy imaging was carried out at predetermined intervals viz. 0.25, 0.5, 1, 2, 3, 4 hours by a Single Photon Emission Computerized Tomography (SPECT, LC 75–005, Diacam, Siemens AG, Earlangan, Germany) gamma camera. Deposition of intravenously administered formulation in whole body was calculated by taking into account gamma camera attenuation, background, and radioactive decay. Lung accumulation of the injected dose was expressed as a percentage of the whole-body deposition.

Two way ANOVA followed by Bonferroni Post-tests was performed in order to reveal the statistical significance. Statistical analysis was done by using GraphPad Prism Version 5.00 for Windows (GraphPad Software, La Jolla, California, USA).

## 8.4 Results and Discussion

### 8.4.1 Hemocompatibility Study

Incubated RBCs dispersion with test substances was observed under microscope. 1% v/v Triton x-100 was used as negative control where the RBCs were ruptured and lost their normal morphology. Whereas, normal saline i.e. 0.9% w/v sodium chloride in water was used as positive control where RBCs were observed to be normal. Standard GCH solution and Standard VLB solution showed some aggregation and rupture of RBCs, mainly because of the pH of plain drug solution [12, 13]. However, GCH loaded Spherulites and VLB loaded Spherulites revealed that RBCs remained unaffected and displayed normal morphology with no signs of aggregation. Figure 8.1 shows that the effect of test substances on RBCs.

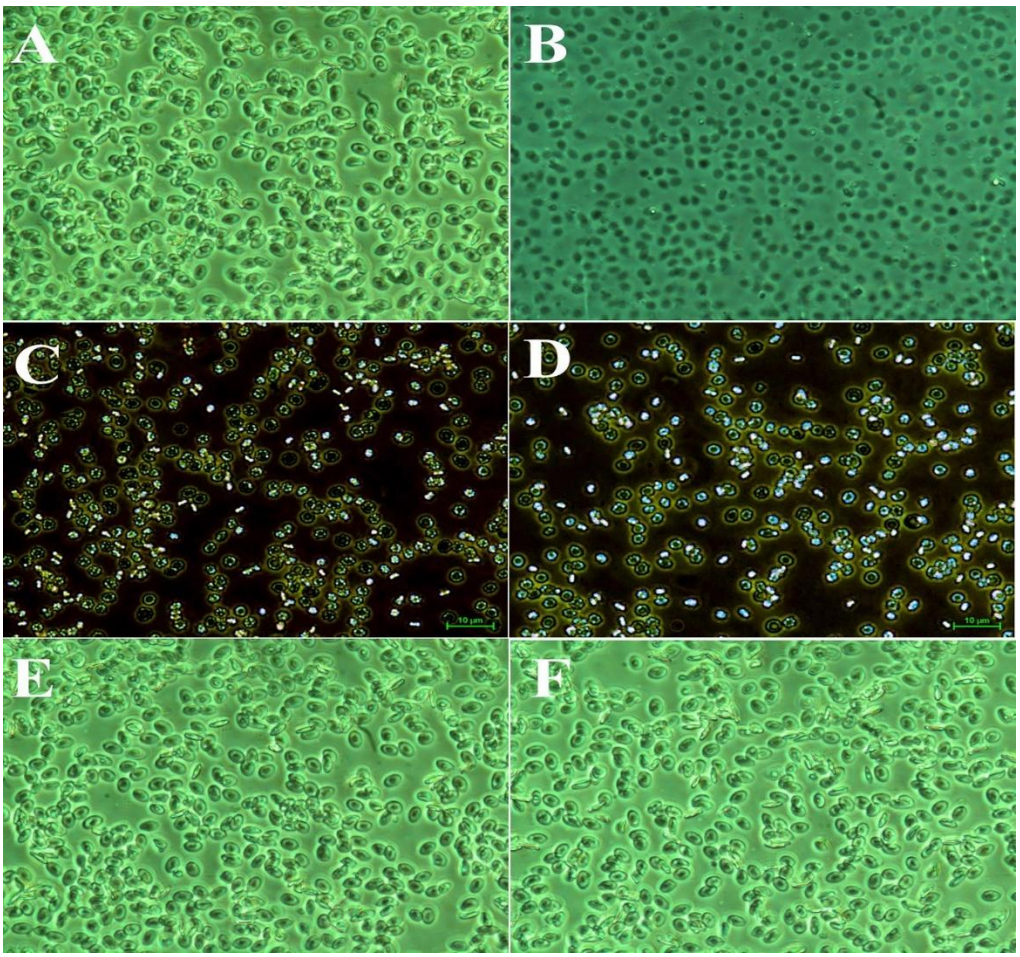


**Figure 8.1:** Microscopic images of RBCs for understanding the compatibility with drug solution and formulation; A) Normal saline, B) 1%v/v Triton x-100, C) Standard GCH solution, D) Standard VLB solution, E) GCH loaded Spherulites and F) VLB loaded Spherulites.

Results of hemocompatibility study indicates that both the formulations i.e. GCH loaded spherulites and VLB loaded spherulites did not showed any hemolysis or coagulation of RBCs. This shows the formulations are safe for administration via intravenous route. Due to change

in pH or tonicity of the formulation RBCs or platelets present in the blood could get coagulated, and the normal morphology of RBCs can get affected [14]. Developed formulations have the pH and osmolality in ideal range which did not affect the blood components, indicating their suitability to administer via intravenous route.

### 8.4.2 Acute Toxicity Study

Standard GCH solution, GCH loaded Spherulites were administered via tail vein in Female Swiss Albino Mice. Selected dosing range was 100, 200, 300, 400 and 500 mg/kg [15, 16]. Animals administered with 100, 200 and 300 mg/kg dose of Standard GCH solution were found healthy and no signs of toxicity was observed. However at a dose of 400 mg/kg, animals showed some signs of toxicity, like after administration, leg weakness till 2 hours was observed. Mortality was observed in animals administered with 500 mg/kg. Thus, 500 mg/kg was found to be the $LD_{50}$ of Standard GCH solution. Reported $LD_{50}$ in mouse is 500 mg/kg [17]. GCH loaded Spherulites showed no signs of toxicity in experimental animals. However, weight loss was observed in animals administered with highest dose i.e. 500 mg/kg. Thus, $LD_{50}$ of GCH loaded Spherulites was found to be > 500 mg/kg, > (greater than) symbol which indicates that the toxicity endpoint being tested was not achievable at the highest dose used in the test.). Results of acute toxicity study clearly indicate that GCH loaded Spherulites were well tolerated than Standard GCH solution at higher dose.

Standard VLB solution and VLB loaded Spherulites were administered via tail vein in Female Swiss Albino Mice. Dosing range was selected as 20, 30, 42, 50, 60 and 75mg/kg. Animals administered with 20 and 30 mg/kg of Standard VLB solution showed no signs of toxicity. However, mortality was observed at 42 mg/kg dose and it was considered as $LD_{50}$ of Standard VLB solution. Reported $LD_{50}$ in mouse is 28 mg/kg [18]. Subsequently, VLB loaded Spherulites were administered intravenously in mice and it was observed that animals tolerated 20, 30, 42 and 50 mg/kg dose without showing any sign of toxicity. At 60 mg/kg of dose, animals showed toxicity signs like weight loss and loss of fur with decreased feeding. However, $LD_{50}$ of VLB loaded Spherulites was observed at 75 mg/kg dose. Results of acute toxicity study revealed that VLB loaded Spherulites showed better tolerance at higher dose than Standard VLB solution.

The results of acute toxicity study indicate that GCH and VLB in spherulites formulation are better tolerated at higher doses than plain drugs. Plain drug solution of cytotoxic agents can affect normal tissues and at high doses they show signs of toxicity or mortality. This gives a rationale to formulate a carrier system loaded with drug to reduce the toxicity.

### 8.4.3 *In vivo* Pharmacokinetic study

Standard GCH solution, GCH loaded non-PEGylated Spherulites and GCH loaded PEGylated Spherulites were administered in rats intravenously via tail vein at concentration of 0.5 mg. Plasma concentration of test substances administered into experimental animal was analyzed by validated LCMS-MS analytical method. A non-compartmental model was applied to calculate the pharmacokinetic parameters shown in Table 8.1. Figure 8.2 depicts drug concentration in plasma with respect to time.

**Table 8.1:** *In vivo* pharmacokinetic parameters of Standard GCH solution, GCH loaded non-PEGylated Spherulites and GCH loaded PEGylated Spherulites administered intravenously via tail vein in Female SD rats (n=6) (Data represents mean±SD).

| Pharmacokinetic Parameters | Standard GCH solution | GCH loaded non-PEGylated Spherulites | GCH loaded PEGylated Spherulites |
|---|---|---|---|
| $C_{max}$ ($\frac{ng}{mL}$) | 483659±5764 | 493263±6473 | 501327±7438 |
| $AUC_{0\text{-}48}$ ($\frac{ng}{mL} * h$) | 1070712±32031.35 | 5075280±121705.89 | 7530701±209665.62 |
| $AUC_{0\text{-}\infty}$ ($\frac{ng}{mL} * h$) | 1139583±50685.21 | 5106103±133418.74 | 7878707±283447.07 |
| $T_{1/2}$ (h) | 3.47±0.30 | 6.56±0.45 | 10.73±0.56 |
| Vd (L) | 0.0016633 | 0.0010042 | 0.0010186 |
| MRT (h) | 3.38±0.31 | 10.04±0.33 | 14.52±0.70 |
| Cl ($\frac{\mu l}{h}$) | 433.33±15.28 | 97.96±2.56 | 63.51±2.28 |

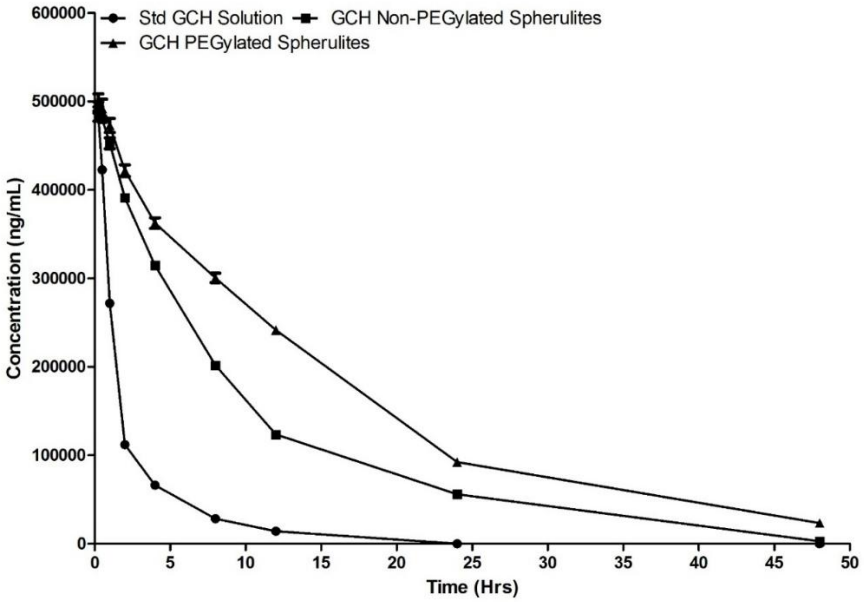


**Figure 8.2:** Plasma concentration Vs. Time profile of Standard GCH solution, GCH loaded non-PEGylated Spherulites and GCH loaded PEGylated Spherulites administered intravenously via tail vein in Female SD rats (n=6). (Data represents mean±SD).

Pharmacokinetic data revealed the difference between the *in vivo* fate of standard GCH solution, GCH loaded non-PEGylated and GCH loaded PEGylated formulation. Table 8.1 and Figure 8.2 shows that there was significant ($p<0.001$) difference in AUC of all three test samples. PEGylated formulation displayed significantly ($p<0.001$) higher AUC followed by non-PEGylated formulation and standard drug solution. GCH loaded non-PEGylated spherulites showed 4.4 fold increase in AUC when compared with standard GCH solution. GCH loaded PEGylated spherulites showed 6.9 fold increase in AUC when compared with GCH loaded non-PEGylated spherulites. Moreover, PEGylated formulation showed increased half-life and mean residence time than non-PEGylated formulation followed by standard drug solution. Also, Clearance rate of PEGylated formulation was much reduced when compared with non-PEGylated formulation followed by standard drug solution.

Standard GCH solution showed $t_{1/2}$ of 3.47 ± 0.30 h which is due to the rapid metabolism of drug in liver and excretion through renal filtration. Encapsulation of GCH in a carrier system protected it from such metabolism and excretion. Whereas, PEGylated formulation exhibited high AUC, long half-life and MRT and slow rate of clearance when compared with non-PEGylated one. PEG coat on spherulites facilitated the long circulation of vesicles, where the non-PEGylated spherulites could be up taken by Reticuloendithelial System (RES) resulting into rapid elimination from systemic circulation [19].

Standard VLB solution, VLB loaded non-PEGylated Spherulites and VLB loaded PEGylated Spherulites were administered in rats intravenously via tail vein at concentration of 0.5 mg. Plasma concentration of test substances administered into experimental animal was analyzed by validated LCMS-MS analytical method. A non-compartmental model was applied to calculate the pharmacokinetic parameters shown in Table 8.2. Figure 8.3 depicts drug concentration in plasma with respect to time.

**Table 8.2:** *In vivo* pharmacokinetic parameters of Standard VLB solution, VLB loaded non-PEGylated Spherulites and VLB loaded PEGylated Spherulites administered intravenously via tail vein in Female SD rats (n=6) (Data represents mean±SD).

| **Pharmacokinetic Parameters** | **Standard VLB solution** | **VLB loaded non-PEGylated Spherulites** | **VLB loaded PEGylated Spherulites** |
|---|---|---|---|
| $C_{max}$ $(\frac{ng}{mL})$ | 513287±6472 | 504167±3182 | 515539±5015 |
| $AUC_{0\text{-}48}$ $(\frac{ng}{mL} * h)$ | 12562802±208421.52 | 13615446±343022.30 | 16318993±257938.32 |
| $AUC_{0\text{-}\infty}$ $(\frac{ng}{mL} * h)$ | 15283412±364721 | 17702866±861591.56 | 33425291±1331189.23 |
| $T_{1/2}$ (h) | 19.91±0.36 | 23.38±1.19 | 50.65±1.66 |
| Vd (L) | 0.0009581 | 0.0009679 | 0.0010919 |
| MRT (h) | 27.65±0.53 | 32.58±1.74 | 72.00±2.60 |
| Cl $(\frac{\mu l}{h})$ | 32.72±0.78 | 28.28±1.38 | 14.97±0.60 |

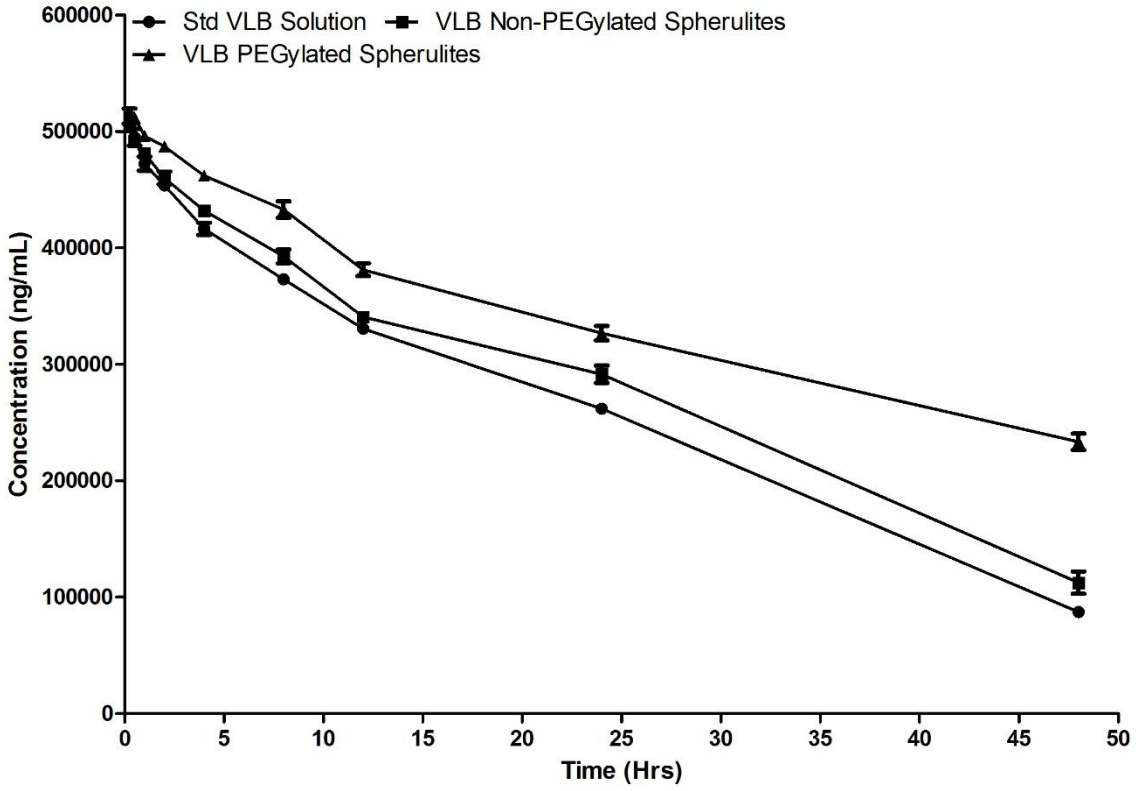


**Figure 8.3:** Plasma concentration Vs. Time profile of Standard VLB solution, VLB loaded non-PEGylated Spherulites and VLB loaded PEGylated Spherulites administered intravenously via tail vein in Female SD rats (n=6). (Data represents mean±SD).

Pharmacokinetic data showed the difference in *in vivo* behavior of standard VLB solution, VLB loaded non-PEGylated and VLB loaded PEGylated formulation. Table 8.2 and Figure 8.3 shows that there was significant ($p<0.001$) difference in AUC of all three test samples. PEGylated formulation displayed significantly ($p<0.001$) higher AUC followed by non-PEGylated formulation and standard drug solution. VLB loaded PEGylated spherulites showed 1.1 fold increase in AUC when compared with VLB loaded non-PEGylated spherulites .Moreover, PEGylated formulation showed increased half-life and mean residence time than non-PEGylated formulation followed by standard drug solution. Also, clearance rate of PEGylated formulation was reduced by one half when compared with non-PEGylated formulation followed by standard drug solution.

Standard VLB solution has inherent long half-life ($t_{1/2}$ 19.91 h) which shows that it remains in systemic circulation [20]. This could be the reason for causing unwanted side effects. Whereas, encapsulation of drug inside the vesicles (PEGylated and non-PEGylated) showed much longer half-life. PEGylation of vesicles enabled them to remain in circulation for longer period of time. Non-PEGylated formulation also remained in circulation for longer time than standard VLB solution but due to the surface property of vesicles they got eliminated much rapidly when compared with PEGylated formulation.

### 8.4.4 *In vivo* Biodistribution study by LCMS-MS analysis

Standard GCH solution, GCH loaded non-PEGylated Spherulites and GCH loaded PEGylated Spherulites were administered in rats intravenously via tail vein at concentration of 0.3 mg. Experimental animals were euthanized and highly perfused organs like Heart, Lungs, Liver, Spleen and Kidneys were isolated. Isolated organs were immediately homogenized and drug extraction procedure was carried out (Chapter 3 Analytical methods). The drug concentration was quanified by validated LCMS-MS method.

Table 8.3 and Figure 8.4 shows the Standard GCH solution disposition in highly perfused organs at various time intervals.

**Table 8.3:** Biodistribution of Standard GCH solution in rats in highly perfused organs as function of time (n=3) (Data represents mean±SD).

| Time (h) | Heart | Lungs | Liver | Spleen | Kidneys |
|---|---|---|---|---|---|
| 0.25 | 0.52±0.29 | 16.53±3.89 | 14.52±2.46 | 3.40±1.24 | 6.74±2.09 |
| 0.5 | 0.47±0.13 | 15.70±2.37 | 11.07±1.83 | 3.83±0.76 | 5.49±1.37 |
| 1 | 0.63±0.19 | 13.27±1.54 | 10.59±2.45 | 4.73±0.96 | 3.58±1.09 |
| 2 | 0.37±0.11 | 6.39±1.87 | 4.41±1.49 | 2.72±0.83 | 4.25±1.76 |
| 4 | 0.57±0.15 | 2.54±0.93 | 1.18±0.46 | 1.68±0.37 | 1.47±0.29 |
| 8 | 0.72±0.21 | 1.15±0.39 | 0.73±0.14 | 1.29±0.57 | 0.69±0.19 |
| 12 | 0.31±0.12 | 0.43±0.19 | 0.24±0.10 | 0.29±0.14 | 0.15±0.09 |

Data represents % dose injected of total dose estimated from organs. (n=3; mean±SD)

As seen from the results Standard GCH solution showed non-specific biodistribution. GCH was found in Heart, Lungs, Liver, Spleen and Kidneys. Initially the drug concentration in all the highly perfused organs except Heart was high, however, in the later time points it declined. GCH has a very short half-life because of its rapid metabolism by hepatic enzymes and excretion through urine. Liver and Spleen collectively called as RES had shown higher drug concentration. The results obtained clearly states that drug gets rapidly eliminated and also, it is evident that non-specific biodistribution of a cytotoxic drug can cause serious side effects.

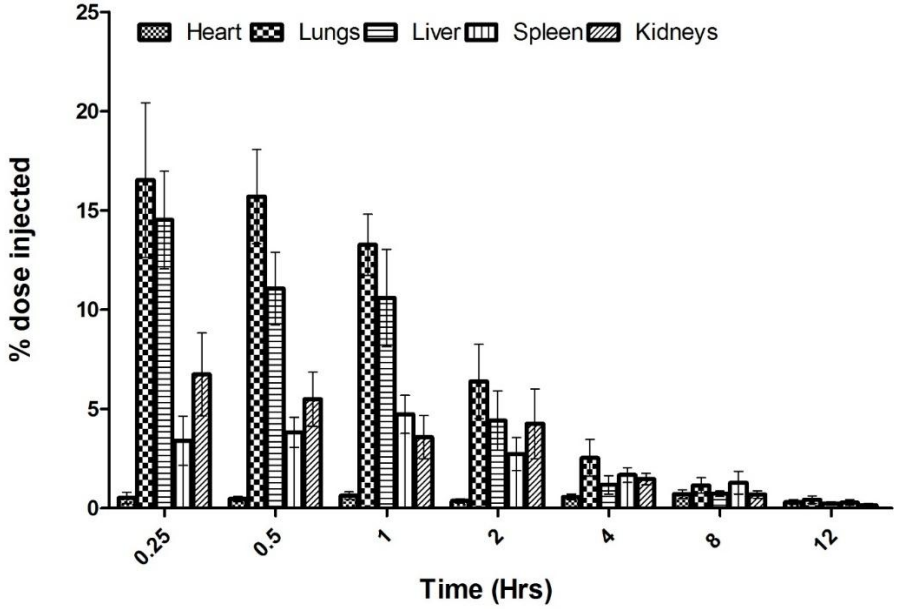


**Figure 8.4:** Biodistribution of Standard GCH solution in rats in highly perfused organs as function of time. Data represents % dose injected of total dose estimated from organs. (n=3; mean±SD).

**Table 8.4:** Biodistribution of GCH loaded non-PEGylated Spherulites in rats in highly perfused organs as function of time (n=3) (Data represents mean±SD).

| Time (h) | Heart | Lungs | Liver | Spleen | Kidneys |
|---|---|---|---|---|---|
| 0.25 | 0.73±0.55 | 9.27±2.91 | 7.29±1.54 | 1.38±0.83 | 10.31±1.36 |
| 0.5 | 0.49±0.13 | 14.64±3.37 | 4.68±1.09 | 0.93±0.58 | 9.07±2.03 |
| 1 | 0.81±0.49 | 11.82±2.63 | 3.90±0.97 | 2.46±1.03 | 6.28±1.69 |
| 2 | 1.45±0.39 | 10.65±2.75 | 2.04±1.65 | 1.93±0.77 | 9.67±1.45 |
| 4 | 0.93±0.27 | 5.94±2.43 | 2.39±0.94 | 2.73±1.31 | 6.70±0.89 |
| 8 | 1.20±0.55 | 2.19±0.79 | 3.65±1.04 | 4.69±0.48 | 3.56±0.72 |
| 12 | 0.67±0.29 | 3.72±1.42 | 7.83±0.67 | 7.12±0.91 | 1.27±0.34 |

Data represents % dose injected of total dose estimated from organs. (n=3; mean±SD)

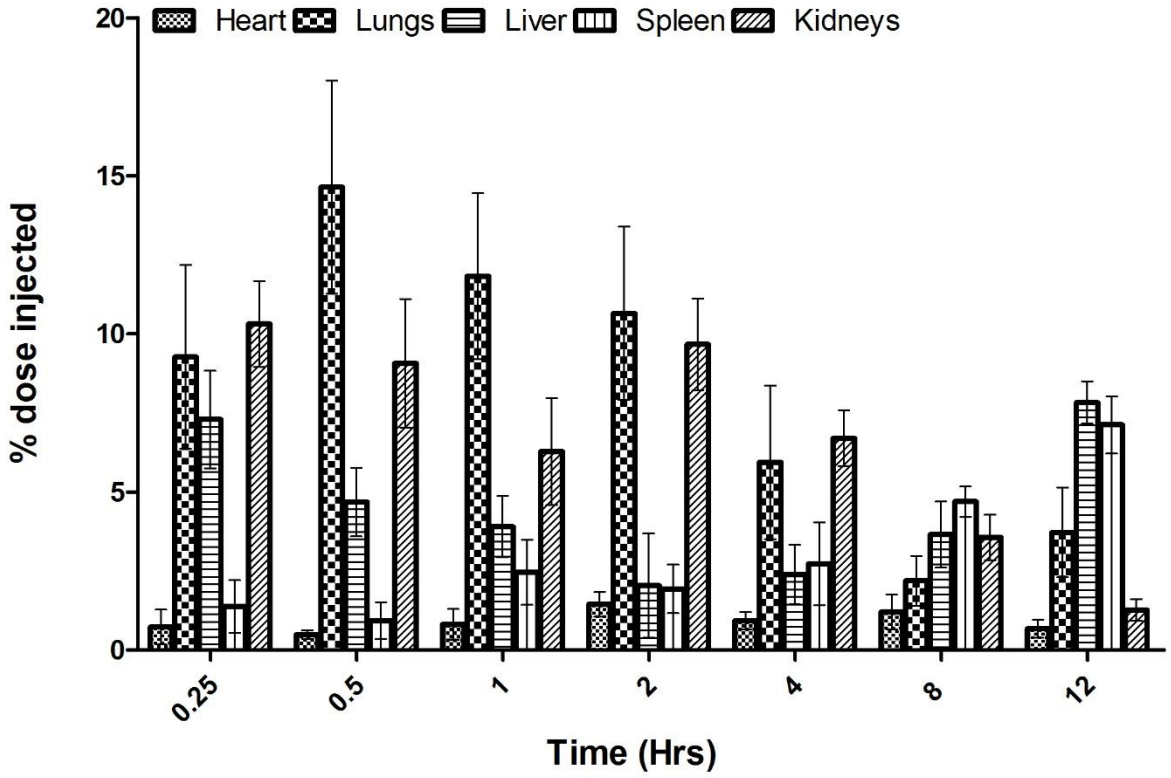


**Figure 8.5:** Biodistribution of GCH loaded non-PEGylated Spherulites in rats in highly perfused organs as function of time. Data represents % dose injected of total dose estimated from organs. (n=3; mean±SD).

Results of Biodistribution of GCH loaded non-PEGylated Spherulites in rats are shown in Table 8.4 and Figure 8.5. GCH loaded non-PEGylated Spherulites displayed that the formulation was reaching to the organ of interest i.e. Lungs, however, it started to decline after 2 hours. Initial accumulation of formulation in Lungs can be seen which shows that Spherulites

could reach the target organ. Another observation was that formulation showed accumulation in Liver, Spleen and Kidneys. This could be because of identification of Non-PEGylated spherulites by RES. RES treats the Non-PEGylated formulation as foreign body and eliminates it from circulation.

**Table 8.5:** Biodistribution of GCH loaded PEGylated Spherulites in rats in highly perfused organs as function of time (n=3) (Data represents mean±SD).

| Time (h) | Heart | Lungs | Liver | Spleen | Kidneys |
|---|---|---|---|---|---|
| 0.25 | 0.89±0.51 | 16.37±3.69 | 5.83±2.07 | 0.51±0.25 | 8.56±2.24 |
| 0.5 | 0.52±0.19 | 18.04±2.59 | 3.65±1.76 | 0.46±0.22 | 7.34±1.83 |
| 1 | 0.75±0.29 | 14.61±1.92 | 2.64±0.85 | 1.07±0.31 | 8.57±1.56 |
| 2 | 1.23±0.64 | 13.57±2.19 | 2.28±1.03 | 0.89±0.45 | 4.27±2.29 |
| 4 | 1.07±0.74 | 7.19±1.74 | 3.83±1.13 | 1.25±0.37 | 2.45±1.22 |
| 8 | 1.34±0.27 | 3.48±1.04 | 2.59±1.43 | 2.67±0.79 | 0.96±0.57 |
| 12 | 0.59±0.32 | 4.32±2.46 | 4.34±1.95 | 3.56±0.93 | 0.58±0.49 |

Data represents % dose injected of total dose estimated from organs. (n=3; mean±SD)

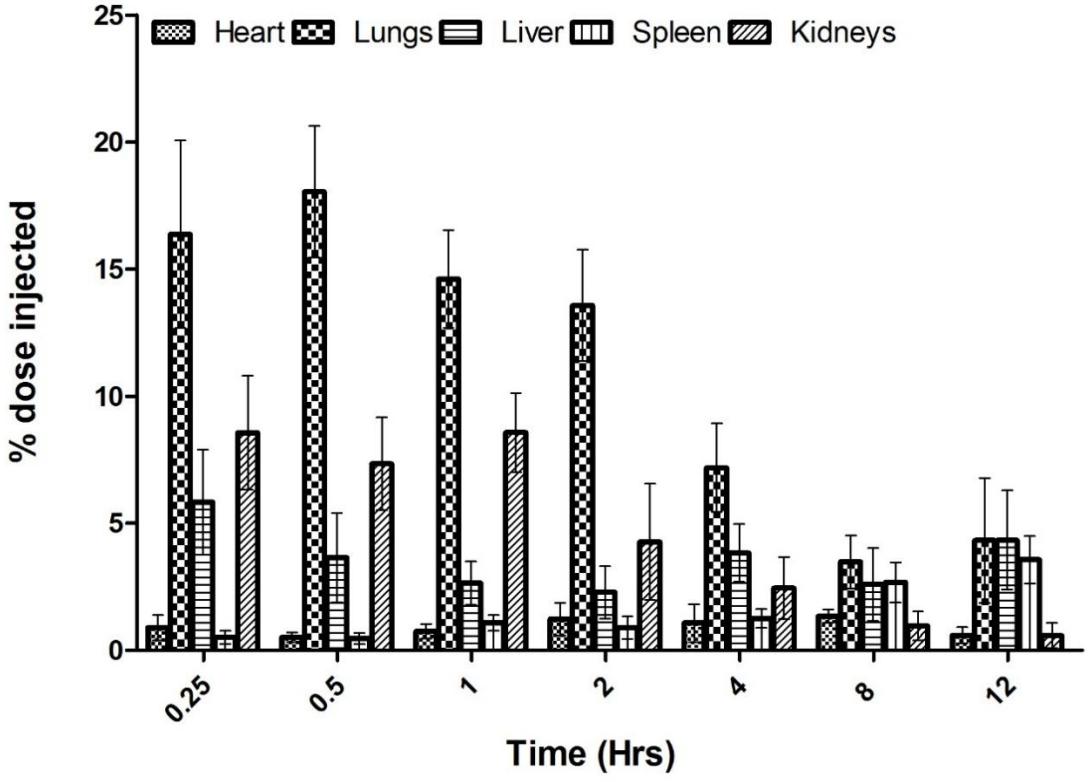


**Figure 8.6:** Biodistribution of GCH loaded PEGylated Spherulites in rats in highly perfused organs as function of time. Data represents % dose injected of total dose estimated from organs. (n=3; mean±SD).

The biodistribution profile of GCH loaded PEGylated Spherulites is shown in Table 8.5 and Figure 8.6. Results showed that PEGylated formulation reached and retained at the target organ i.e. Lungs till 12 hours. Liver, Spleen and Kidney showed minimal drug disposition. This could be due to the presence of PEG chains over the vesicles surface enabling them to remain undetected from RES.

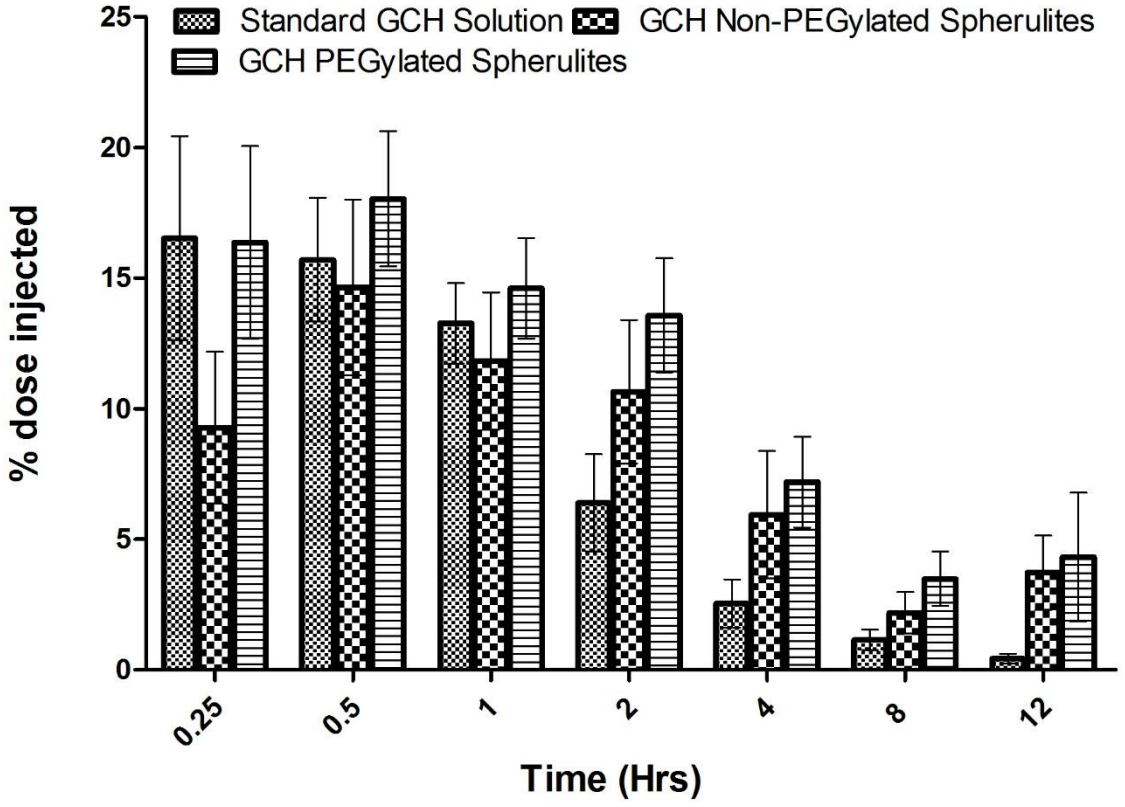


**Figure 8.7:** Comparison of lung targeting potential of Standard GCH Solution, GCH loaded Non PEGylated spherulites and GCH loaded PEGylated spherulites. Data represents % dose injected of total dose estimated from Lungs. (n=3; mean±SD) (Statistically analyzed by Two way ANOVA; $p<0.0001$).

Lung targeting of all three test substances viz. Standard GCH Solution, GCH loaded Non PEGylated spherulites and GCH loaded PEGylated spherulites is shown in Figure 8.7. Results indicate that standard GCH showed disposition in Lungs initially, however, as the time progressed, it declined. This was due to the reason that plain drug is prone to metabolism and excretion. Also, short half-life of Standard GCH is an accountable reason for rapid elimination. Although it reached Lungs in higher amount till 1 hour, however, other highly perfused organs like Liver, Spleen and Kidneys also showed increased disposition which was increasing with the time. This indicates that drug got distributed variedly throughout the body with increase in

chance of adverse effects. Whereas, PEGylated and Non-PEGylated formulation of GCH remained in the circulation for longer period of time. However, GCH loaded Non PEGylated spherulites showed less disposition in target organ than PEGylated formulation. Non-PEGylated formulation might be taken up by RES and hence, eliminated from systemic circulation. PEGylation of GCH loaded Spherulites was performed by adding 2 mol% of DSPE-PEG 2000. Hydrophilic chains of PEG over the surface of vesicles enabled them to remain in circulation for longer period of time and helped to accumulate in Lungs passively.

Standard VLB solution, VLB loaded non-PEGylated Spherulites and VLB loaded PEGylated Spherulites were administered in rats intravenously via tail vein at concentration of 0.3 mg. Experimental animals were euthanized and highly perfused organs like Heart, Lungs, Liver, Spleen and Kidneys were isolated. Isolated organs were immediately homogenized and drug extraction procedure was carried out (Chapter 3 Analytical methods). The drug concentration was quanified by validated LCMS-MS method.

Table 8.6 and Figure 8.8 shows the standard VLB solution disposition in highly perfused organs at various time intervals.

**Table 8.6:** Biodistribution of Standard VLB solution in rats in highly perfused organs as function of time (n=3) (Data represents mean±SD).

| Time (h) | Heart | Lungs | Liver | Spleen | Kidneys |
|---|---|---|---|---|---|
| 0.25 | 2.73±0.52 | 11.64±1.72 | 9.64±1.07 | 3.42±0.68 | 7.61±0.79 |
| 0.5 | 3.40±0.83 | 15.93±2.86 | 12.56±1.59 | 2.91±1.14 | 9.16±2.19 |
| 1 | 2.07±0.67 | 14.62±2.31 | 12.23±1.97 | 6.77±1.20 | 11.52±2.47 |
| 2 | 2.24±1.29 | 12.59±2.63 | 14.15±3.09 | 10.39±2.48 | 9.95±1.78 |
| 4 | 4.51±1.04 | 11.73±2.27 | 13.61±2.65 | 9.86±1.84 | 10.28±2.56 |
| 8 | 3.67±1.46 | 12.36±2.86 | 11.86±1.91 | 10.49±2.04 | 11.07±1.75 |
| 12 | 2.19±0.87 | 10.45±2.37 | 9.74±1.57 | 12.63±2.19 | 10.93±1.64 |

Data represents % dose injected of total dose estimated from organs. (n=3; mean±SD)

Results shown in Table 8.6 and Figure 8.8 indicates that standard VLB solution showed a wide spread biodistribution i.e. non-specific. VLB was found in Heart, Lungs, Liver, Spleen and Kidneys. VLB concentration in all the isolated highly perfused organs was found to be persistent from 0.25 hour to 12 hours. The reason for this is that VLB has long half-life as a results of which it gets accumulated in highly perfused organs. Consequently, presence of a cytotoxic agent in systemic circulation for longer period of time can cause side effects.

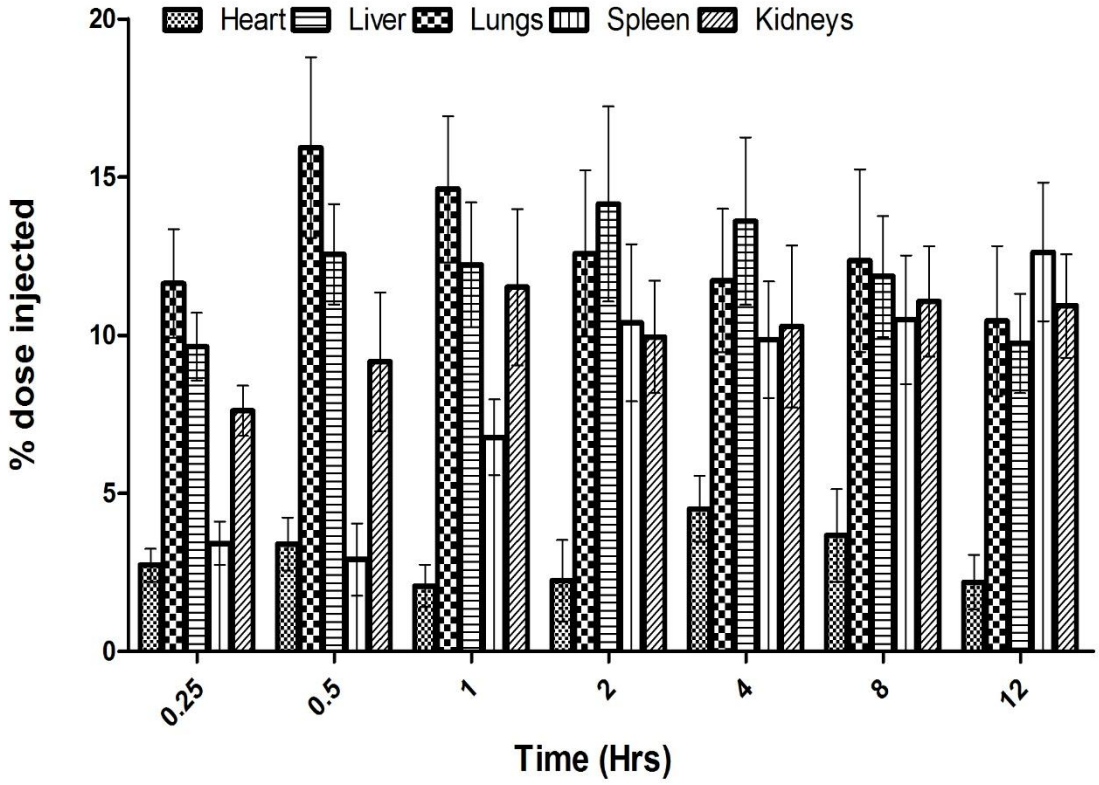


**Figure 8.8:** Biodistribution of Standard VLB solution in rats in highly perfused organs as function of time. Data represents % dose injected of total dose estimated from organs. (n=3; mean±SD).

**Table 8.7:** Biodistribution of VLB loaded non-PEGylated Spherulites in rats in highly perfused organs as function of time (n=3) (Data represents mean±SD).

| Time (h) | Heart | Lungs | Liver | Spleen | Kidneys |
|---|---|---|---|---|---|
| 0.25 | 1.09±0.63 | 7.94±1.87 | 4.83±1.42 | 2.27±0.76 | 12.59±1.54 |
| 0.5 | 0.79±0.34 | 13.42±1.61 | 7.59±2.54 | 1.95±0.77 | 10.38±1.97 |
| 1 | 1.37±0.41 | 10.09±2.27 | 5.91±1.32 | 4.09±2.04 | 8.42±1.86 |
| 2 | 2.03±1.16 | 9.76±1.82 | 4.28±1.51 | 3.48±0.94 | 10.07±2.49 |
| 4 | 1.54±0.37 | 7.59±1.78 | 4.73±1.32 | 5.34±2.15 | 6.45±1.64 |
| 8 | 1.05±0.57 | 4.27±1.03 | 5.85±1.66 | 6.74±2.43 | 4.89±1.18 |
| 12 | 0.87±0.41 | 2.94±0.84 | 7.90±1.29 | 9.14±1.87 | 2.89±1.02 |

Data represents % dose injected of total dose estimated from organs. (n=3; mean±SD)

Results in Table 8.8 and Figure 8.9 shows the biodistribution pattern of VLB loaded non-PEGylated Spherulites. VLB loaded non-PEGylated Spherulites showed increased lung accumulation till 1 hour, however, it started to decline at later time points. This result indicates

that the formulation is able to reach target organ i.e. Lungs at initial time points. Moreover, the results showed that the non-PEGylated formulation was found to be accumulated in Liver, Spleen i.e. RES system and Kidneys and it was found to be increasing with time. Non-PEGylation of Spherulites could be responsible for the uptake by RES system and consequently elimination from systemic circulation.

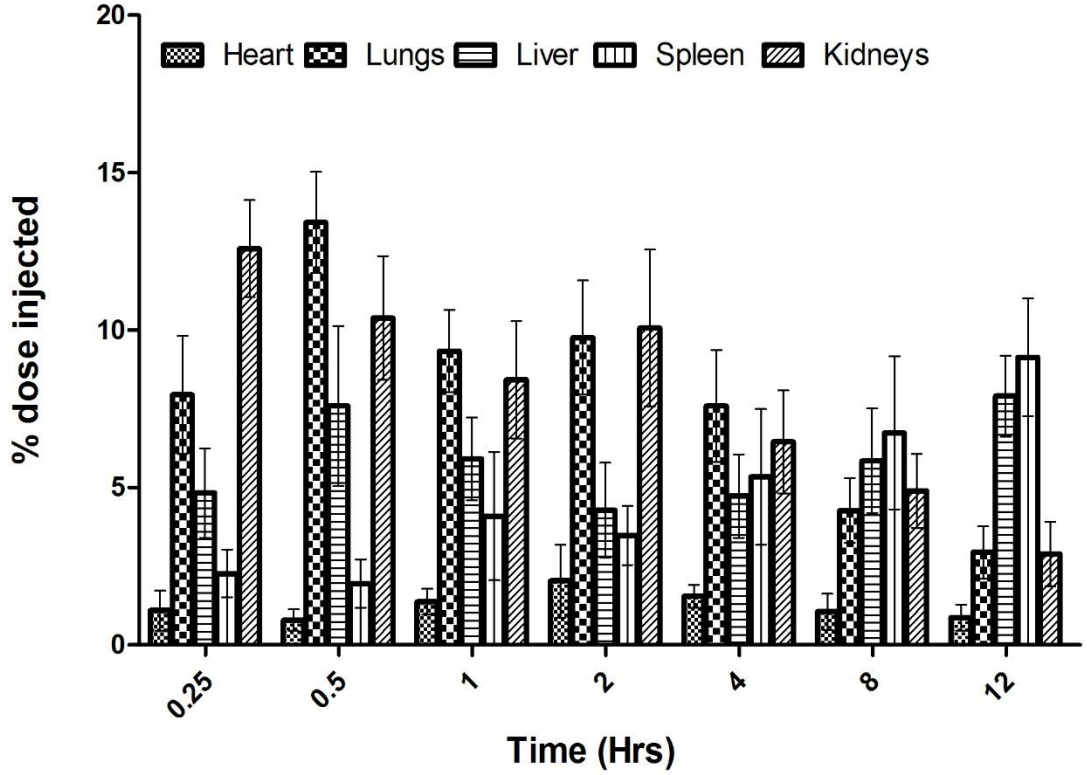


**Figure 8.9:** Biodistribution of VLB loaded non-PEGylated Spherulites in rats in highly perfused organs as function of time. Data represents % dose injected of total dose estimated from organs. (n=3; mean±SD).

**Table 8.8:** Biodistribution of VLB loaded PEGylated Spherulites in rats in highly perfused organs as function of time (n=3) (Data represents mean±SD).

| Time (h) | Heart | Lungs | Liver | Spleen | Kidneys |
|---|---|---|---|---|---|
| 0.25 | 0.97±0.49 | 17.92±3.21 | 3.58±1.06 | 0.82±0.34 | 9.37±1.72 |
| 0.5 | 1.21±0.57 | 19.56±2.85 | 4.75±1.14 | 1.09±0.63 | 8.03±1.38 |
| 1 | 0.84±0.29 | 15.79±1.83 | 3.18±0.58 | 1.32±0.45 | 7.49±1.04 |
| 2 | 0.69±0.37 | 13.81±2.68 | 2.73±1.24 | 0.84±0.19 | 5.32±1.36 |
| 4 | 1.32±0.41 | 11.68±2.07 | 3.15±1.39 | 1.19±0.43 | 3.76±1.19 |
| 8 | 0.84±0.51 | 8.97±1.89 | 2.44±0.63 | 1.47±0.31 | 1.89±0.74 |
| 12 | 1.05±0.67 | 6.27±2.34 | 3.34±1.24 | 2.67±0.83 | 0.94±0.29 |

Data represents % dose injected of total dose estimated from organs. (n=3; mean±SD)

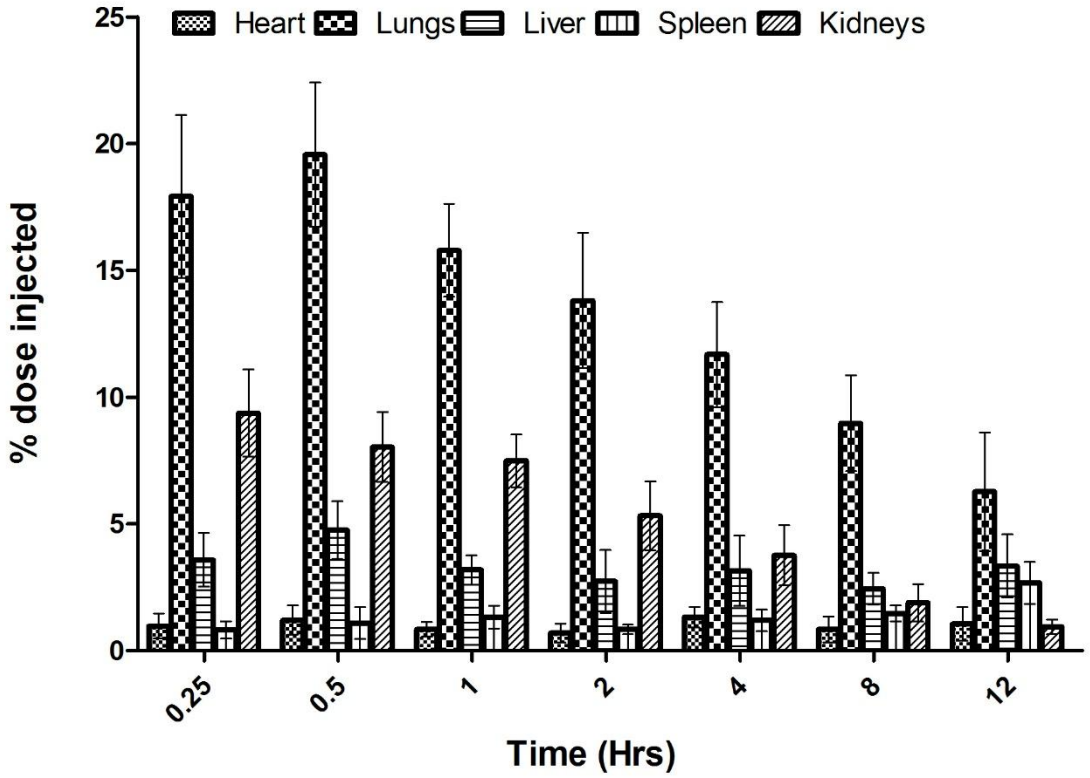


**Figure 8.10:** Biodistribution of VLB loaded PEGylated Spherulites in rats in highly perfused organs as function of time. Data represents % dose injected of total dose estimated from organs. (n=3; mean±SD).

Biodistribution profile PEGylated Spherulites bearing VLB is shown in Table 8.8 and Figure 8.10. Results indicates that the VLB loaded PEGylated Spherulites reached and retained in the Lungs i.e. target organ at higher concentration than other organs till 12 hours. Moreover, the PEGylated Spherulites showed minimal disposition in Liver, Spleen and Kidneys. The attributable reason for this could be the PEGylation of vesicles which enabled the formulation to remain in the circulation for longer period of time by bypassing the RES system.

Lung targeting potential of all three test substances viz. Standard VLB Solution, VLB loaded Non PEGylated spherulites and VLB loaded PEGylated spherulites is shown in Figure 8.11. Standard VLB Solution showed persistent concentration in Lungs from initial to later time points. Although, plain drug reached the lungs in high concentration than both the formulations, but at the same time other highly perfused organs like Liver, Spleen and Kidneys were also found to have persistent drug accumulation. This is known as non-specific biodistribution of Standard VLB Solution. Moreover, VLB has a long half-life which might be another reason for its accumulation in lungs. Consequently, plain drug solution causes side effects over other healthy tissues. Whereas, VLB loaded Non PEGylated spherulites stayed in the circulation and

showed targeting to lungs at increasing concentration till 2 hours. However, it started declining at later points due to RES uptake. VLB loaded PEGylated spherulites efficiently got localized in lungs at higher concentration than Non-PEGylated Spherulites and Standard VLB Solution till 12 hours. PEGylation played a crucial role for formulation to be retained in the circulation and passive accumulation in Lungs.

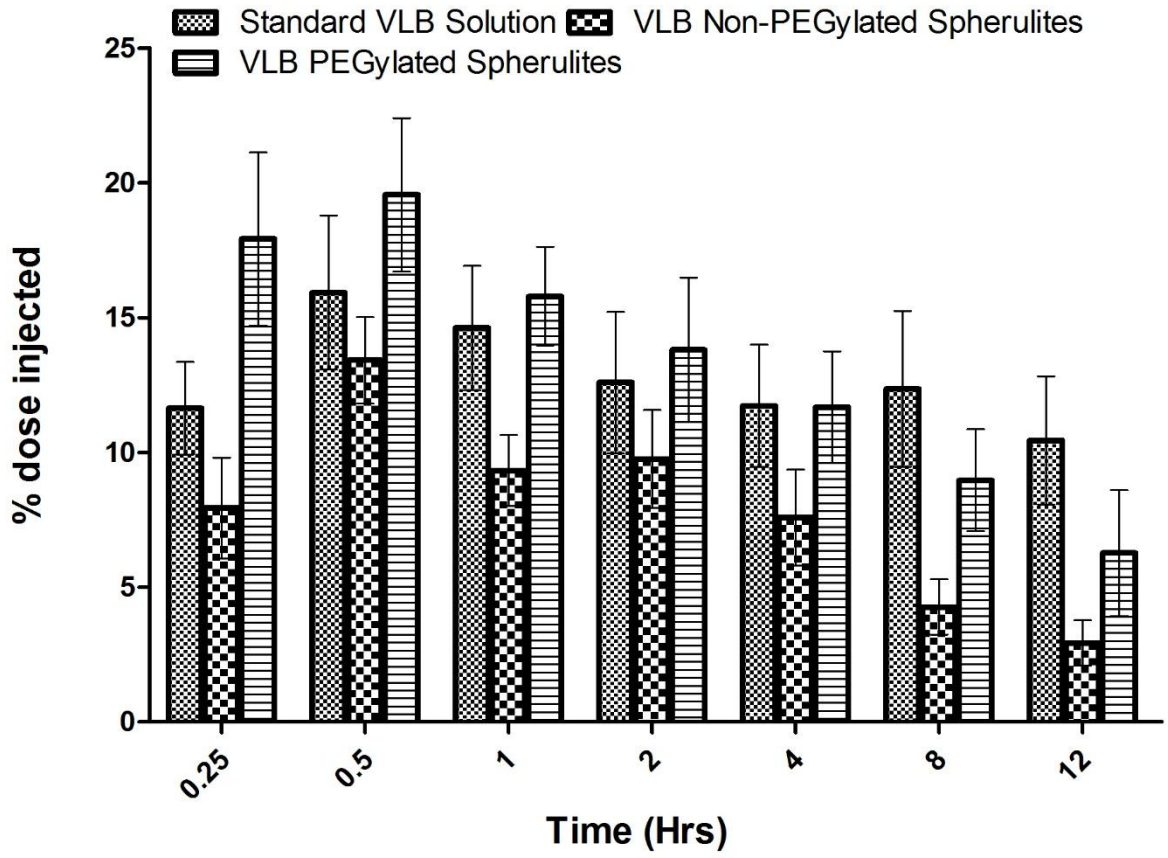


**Figure 8.11:** Comparison of lung targeting potential of Standard VLB Solution, VLB loaded Non PEGylated spherulites and VLB loaded PEGylated spherulites. Data represents % dose injected of total dose estimated from Lungs. (n=3; mean±SD) (Statistically analyzed by Two way ANOVA; $p<0.0001$).

### 8.4.5 *In vivo* Biodistribution study of GCH and GCH loaded formulations by Gamma (γ) scintigraphy [10]

#### 8.4.5.1 Radiolabelling, Quality Control, and stability of $^{99m}$Tc-GCH

Sodium pertechnetate is obtained from Molybdenum as the chemical form of 99mTc. TcO-4 is the pertechnetate ion and has oxidation state of +7, this ion is unable to form complex with any compound upon direct addition because of its non-reactive nature. 99mTc is required to be reduced from +7 state to a much lower state for efficient labelling of compounds. Stannous

chloride is the widely used $^{99m}$Tc reducing agent, however, Sn+2 ion in stannous chloride undergoes hydrolysis in presence of aqueous solution at pH 6-7 forming insoluble colloids. 99mTc binds with these colloids and thus the labeling yield is compromised. Due to this reason, to prevent the hydrolysis of Sn+2 an acid is added just before the reduction of technetium. The mean labeling efficiency of GCH was >98% at pH 6.5 (adjusted using 0.5M $NaHCO_3$ solution). Less than 1% radioactivity was dissociated after 24 hours incubation.

$^{99m}$Tc-GCH complex was incubated in human serum and 0.9% saline at 37 $^{o}$C, results showed that the complex was stable (Table 8.9). Radiolabelled complex upon 24 hours incubation in serum and saline dissociated less than 1% to 3% indicating the suitable stability of the complex for its *in vivo* use.

**Table 8.9:** $^{99m}$Tc-GCH complex stability in serum and saline (n=3; mean±SD).

| Time (Hours) | 0.9% Saline | Human Serum |
|---|---|---|
| 0 | 97.48±3.13 | 97.02±1.42 |
| 0.5 | 98.28±2.14 | 96.21±2.35 |
| 1 | 98.08±2.55 | 96.69±2.21 |
| 2 | 98.78±1.59 | 97.92±1.89 |
| 4 | 99.14±0.86 | 98.02±1.43 |
| 6 | 99.30±0.94 | 98.08±1.56 |
| 24 | 99.71±0.19 | 99.40±1.16 |

### 8.4.5.2 Transchelation Study (DTPA Challenge)

The binding strength of technetium with GCH was evaluated by DTPA challenge study. The results of DTPA challenge study showed that DTPA present in the solution did not alter the labeling efficiency (Figure 8.12). At highest of 7.5 mM concentration of DTPA, it was found that the transchelation confirmed by ITLC was less than 3.5%, this indicated high stability of the radiolabeled complexes. DTPA has greater affinity towards $^{99m}$Tc. Incubation of labelled complex in presence of DTPA results in transchelation. Less stable complex shows high degree of transchelation, whereas, complex with high stability indicates low degree of transchelation.

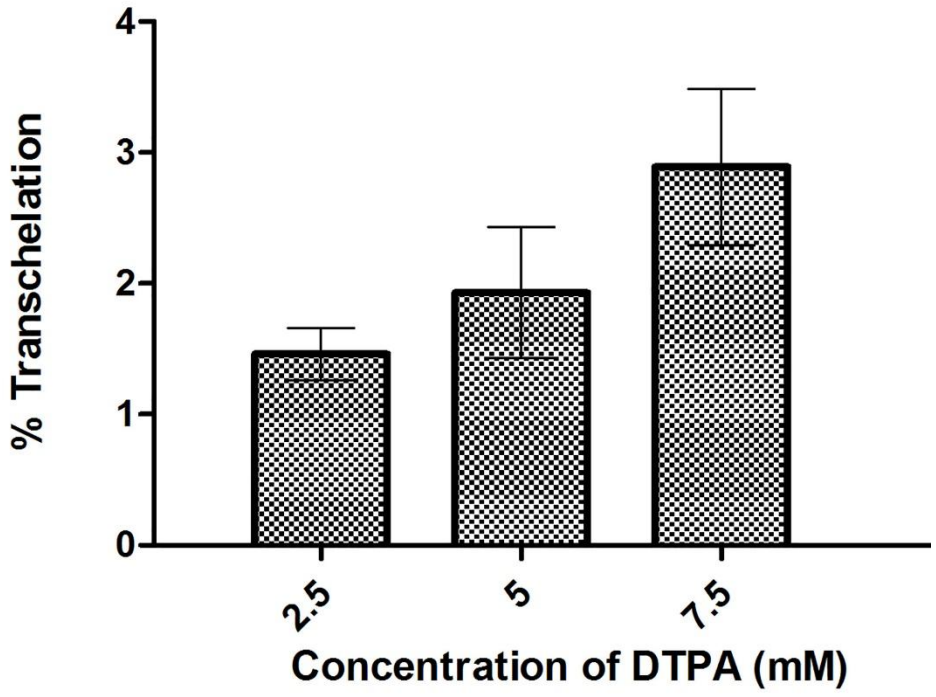


**Figure 8.12:** $^{99m}$Tc-GCH complex DTPA challenge study (n=3; mean±SD).

**8.4.5.3 *In vivo* Biodistribution studies in Rats**

Targeted drug therapies have emerged because of the parallel development of technological advancements and thorough understanding of the molecular and cellular biology of cancer. The aim of the current investigation was to evaluate the targeting efficiency of drug-loaded spherulites to non-small cell lung cancer. Biodistribution of both $^{99m}$Tc-labelled GCH-loaded in spherulites as well as $^{99m}$Tc-labelled GCH plain drug was studied in SD rats by administering the required dose intravenously through tail vein. Nuclear medicine procedures provide a unique tool to study the pharmacodynamics (what the drug does to the body) and pharmacokinetics (what the body does to the drug) noninvasively in the body in real time. Gamma Camera imaging provides data and images of the radiopharmaceutical's distribution in the body and gives diagnostic information about the target organs. Over the last few years, this functional imaging technique has been utilized in evaluation of novel drug delivery systems including the nanostructured ones. It provides a rapid and accurate means of predicting *in vivo* behavior of drugs including their evaluation at preclinical and clinical stages of development.

As seen from the results in Figure 8.13 (A1-F1) and 8.15 (A), $^{99m}$Tc- labeled GCH plain drug was variedly distributed throughout the body of rat with high initial concentrations present in extensively perfused organs like Liver, Kidneys and Lungs. Plain drug solution was rapidly cleared from the blood circulation except some localization seen in kidney. The probable

reason for this phenomenon is due to the metabolism of the drug and its rapid clearance by the urine, as a result leading to a poor localization at site of action i.e. Lungs. Whereas, in the case of $^{99m}$Tc- labeled GCH loaded in Spherulites, both non-PEGylated and PEGylated formulations remained in circulation for a longer period of time owing to the characteristics of the vesicular system as seen in Figures 8.13 (A2-F2) and 8.14. In addition, localization of Spherulites in to lungs was significantly higher compared to plain drug and it remained detectable for a longer period of time. The initial distribution, within 1 hour of administration, of PEGylated Spherulites to lungs was significantly higher (18.73%, Figure 8.15C) than that of Non PEGylated (12.17%, Figure 8.15B) and plain drug solution (14.62%, Figure 8.15A) as seen in Figure 8.15. As observed in the Figure 8.16, even after 4 hours of administration, lung retention of PEGylated Spherulites (9.11%) exceeded that of non-PEGylated (4.31%) and plain drug solution (1.20%). The reason for this is the uptake of free drug by the RES system leading to its metabolism in liver whereas, when the drug is entrapped in to the Spherulites, it gets stabilized and protected by the lipidic bilayers. Moreover, attachment of PEG chains on the surface of the Spherulites i.e. PEGylation/Stealth provides steric stabilization of vesicles which increases their longevity in circulation. The space adjacent to vesicle surface is immediately occupied by the flexible chain of PEG, a hydrophilic polymer, which avoids other macromolecules from this space. These PEG chains create hindrance to the entry and binding of blood plasma macromolecules often regarded as opsonins to the surface of Spherulites, which results in less interaction of macrophages with formulation.

Passive accumulation of long-circulating Spherulites inside tissues or organs can be achieved by reducing the phagocytic uptake. This phenomenon, regarded as passive targeting, is mainly observed in solid tumors going through the phase of angiogenesis: leaky epithelial lining present in the tumor vasculature during angiogenesis promotes the accumulation of vesicular formulations, moreover, the poor lymphatic drainage in tumor microenvironment allows the formulation to remain there for more time and function as a sustained drug-delivery system. This is referred as a well-known phenomenon of Enhanced permeation and retention (EPR) effect. EPR mechanism considered to be responsible in improved efficacy of anticancer drug entrapped in to vesicles than that of free drug. Different ways can be applied to attach PEG chains on the surface of Spherulites, however, most acceptable method is to use the cross-linked lipid (i.e., PEG-distearoylphosphatidylethanolamine [DSPE) block copolymer. Vesicular membranes when anchored with PEG-lipid, forms an aqua-compatible layer around the vesicles surface due to the localization of PEG chains. This process of surface PEGylation

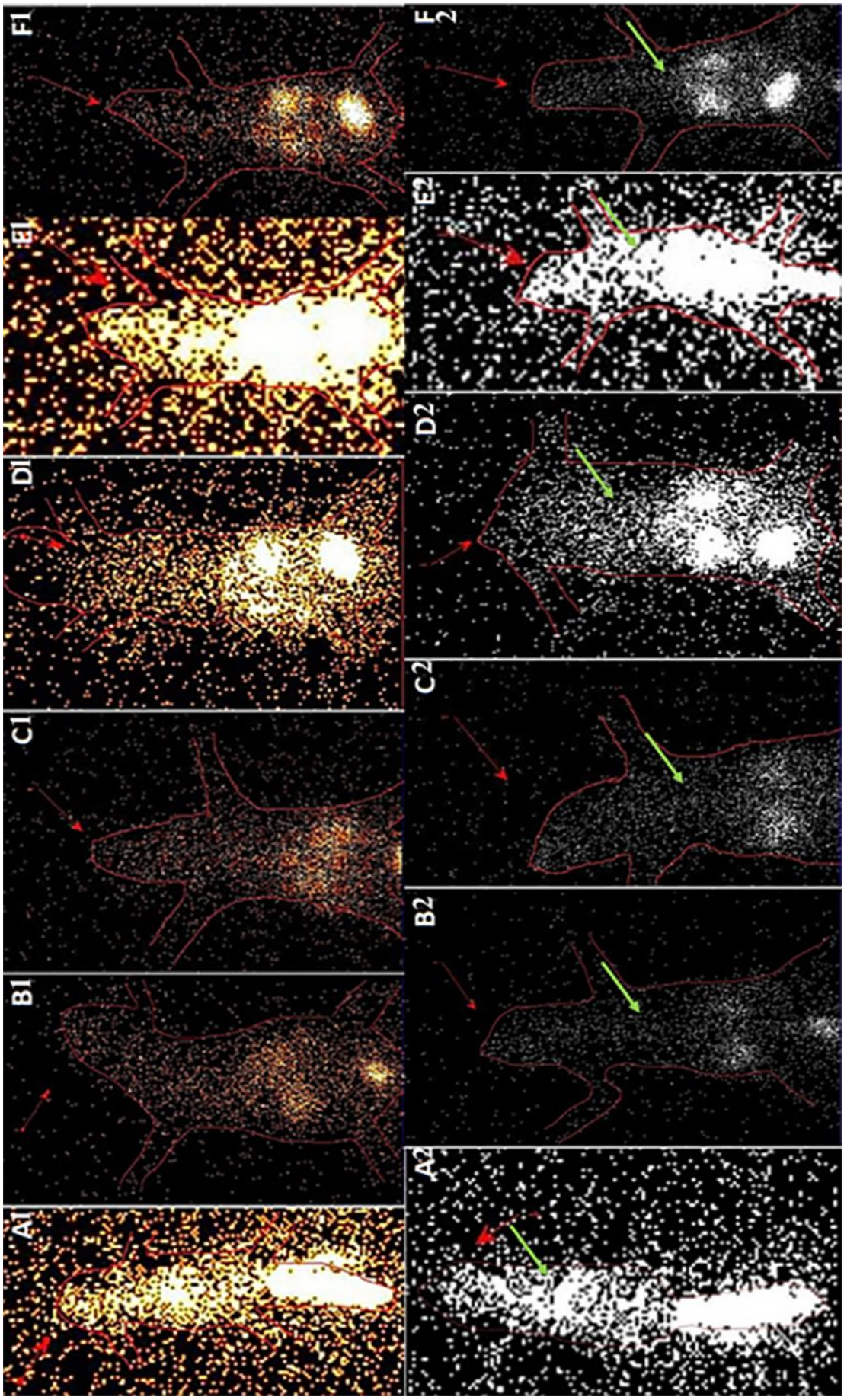


**Figure 8.13** Gamma scintigraphy images at different time points viz. A1) 0.25 hr, B1) 0.5 hr, C1) 1 hr, D1) 2 hrs, E1) 3 hrs, F1) 4 hrs, of rat administered with $^{99m}$Tc labelled GCH plain drug solution showing non specific distribution of plain drug evidenced by scattered gamma quanta and A2) 0.25 hr, B2) 0.5 hr, C2) 1 hr, D2) 2 hrs, E2) 3 hrs, F2) 4 hrs, of rat administered with $^{99m}$Tc labelled GCH loaded Non-PEGylated Spherulites depicting distribution of the formulation.

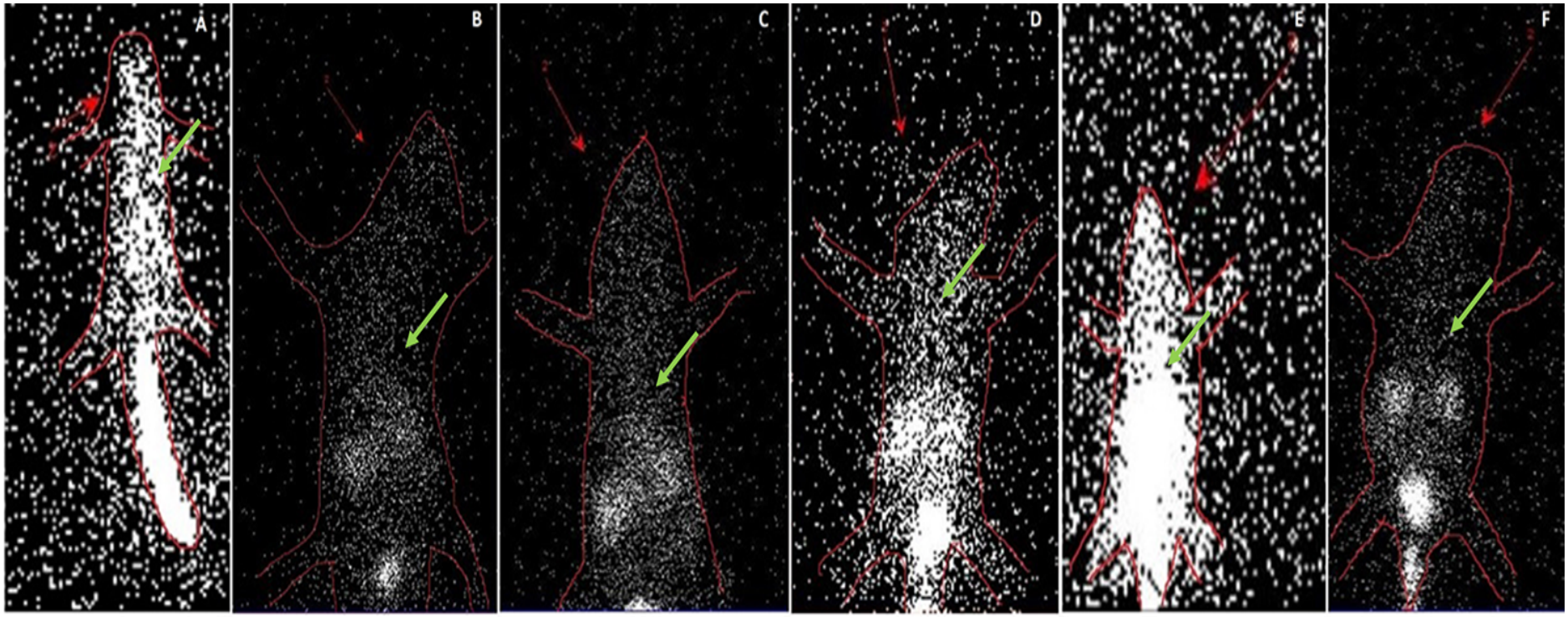


**Figure 8.14:** Gamma scintigraphy images at different time points viz. A) 0.25 hr, B) 0.5 hr, C) 1 hr, D) 2 hrs, E) 3 hrs, F) 4 hrs, of rat administered with $^{99m}$Tc labelled GCH loaded PEGylated Spherulites showing targeting as well as retention of the formulation at the desired site i.e. lungs (region of interest marked by arrows).

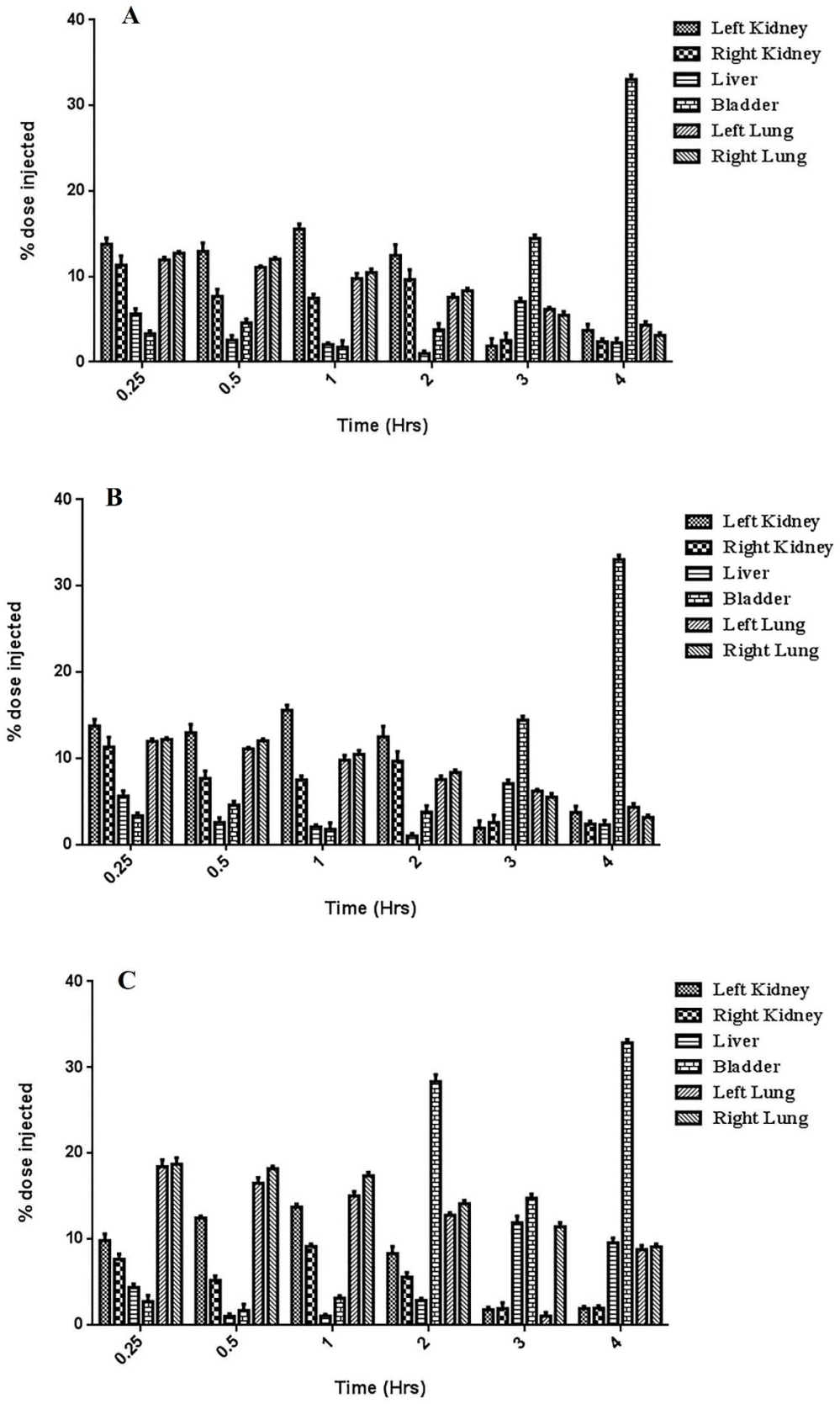


**Figure 8.15:** Biodistribution of (A) $^{99m}$Tc labelled GCH plain drug solution (B) $^{99m}$Tc labelled GCH loaded Non-PEGylated Spherulites and (C) $^{99m}$Tc labelled GCH loaded PEGylated Spherulites in rats in different organs as function of time.

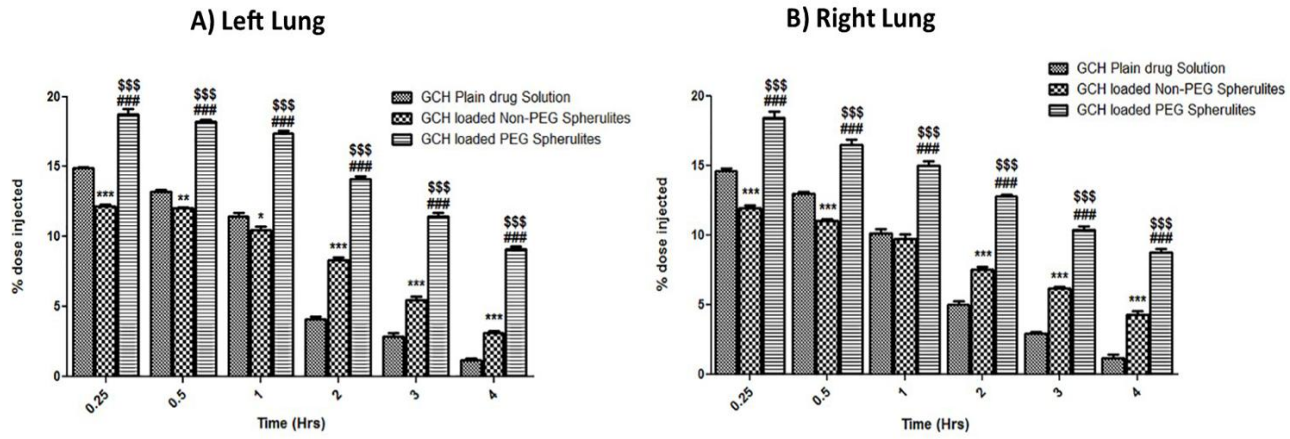


**Figure 8.16:** Comparison of lung targeting potential of GCH plain drug, GCH loaded Non-PEgylated spherulites and GCH loaded PEGylated spherulites, where: A) Left Lung, B) Right Lung. (Where, For Plain drug Solution Vs. Non-PEGylated Spherulites; *** $p<0.0001$, ** $p<0.01$ and * $p< 0.05$. For Plain drug Solution Vs. PEGylated Spherulites; ### $p<0.0001$. For Non-PEGylated Spherulites Vs. PEGylated Spherulites; $$$ $p<0.0001$).

avoids the vesicles to be identified by opsonins (i.e., antibodies or components enhancing phagocytosis) resulting in reduction in the clearance of the vesicles by RES. Blood circulation time for PEG-vesicles significantly increased (i.e., t1/2 >40 h) with even distribution throughout the organs. Distribution of targeted vesicular system takes place in the central compartment (i.e., the blood), reducing the RES uptake 10% to 15% only. About 80% to 90% of conventional non-PEGylated vesicles are taken up by liver, this shows the significant improvement by PEG-vesicles. PEG tethering over the surface of vesicles can be carried out by methods such as: physical adsorption of polymer onto the vesicular surface, incorporation of PEG-lipid block copolymer in formulation step or covalent attachment of reactive groups over the surface of preformed vesicles.

### 8.4.6 *In vivo* Biodistribution study of VLB and VLB loaded formulations by Gamma (γ) scintigraphy [11]

#### 8.4.6.1 Radiolabelling, Quality Control, and stability of $^{99m}$Tc-VLB

Technetium-99m ($^{99m}$Tc) offers many applications in diagnostic, pharmaceutical and biological fields with an advantage of lesser side effects related to radioactivity exposure. $^{99m}$Tc is an ideal radioisotope as, it has short half-life ($t_{1/2}$) of 6 hours and it emits monochromatic gamma radiations of 140 kev strength. Adding to the above, technetium has the chemistry to form a

complex with wide-ranging compounds which shows biological activity and gives promising results in reaching the interested tissues or organs.

Pharmacokinetics and pharmacodynamics are two major studies which demonstrate the fate of an innovative formulation *in vivo*. Nuclear imaging by gamma scintigraphy offers an advantage to examine both these processes noninvasively in real time. Gamma scintigraphy facilitates to understand the *in vivo* behavior of drugs and formulations at early stage of preclinical study as well as in later stage of clinical development.

Labeling efficiency of $^{99m}$Tc-VLB complex was accessed by ITLC. Results stated in Table 8.10 shows that after 24 hours not more than 3% radioactive complex with drug was dissociated. VLB labelled with $^{99m}$Tc was incubated human serum and 0.9% saline at 37 $^{o}$C for 24 hours and it was found that a stable complex is formed. It was found that $^{99m}$Tc-VLB complex was stable for 24 hours with not more than 3% dissociation, showing its suitability for *in vivo* application.$^{99m}$Tc is the chemical form of Sodium pertechnetate obtained from Molybdenum. Pertechnetate ion ($TcO^{-}_{4}$) has oxidation state of +7, as it is non-reactive chemically, complex cannot be formed upon direct addition. Hence, it is necessary to reduce $^{99m}$Tc to much lower state than +7 to enable labelling of active compounds. Commonly Stannous chloride is used as reducing agent for $^{99m}$Tc, yet, the limitation is that $Sn^{+2}$ ion in presence of aqueous solution at pH 6-7 tends to get hydrolyzed resulting in formation of insoluble colloids. These colloids have an affinity towards $^{99m}$Tc which consequently reduces the labelling efficiency. Due to this reason before reduction of technetium an acid is added to inhibit the hydrolysis of $Sn^{+2}$ ion.

**Table 8.10:** $^{99m}$Tc-VLB complex stability in serum and saline (n=3; mean±SD).

| Time (Hours) | 0.9% Saline | Human Serum |
|---|---|---|
| 0 | 98.20±1.1 | 98.30±0.42 |
| 0.5 | 98.80±0.87 | 98.60±0.59 |
| 1 | 98.02±1.0 | 99.02±0.61 |
| 2 | 99.60±0.52 | 98.90±0.89 |
| 4 | 99.55±0.98 | 98.80±0.43 |
| 6 | 99.68±0.87 | 97.01±0.56 |
| 24 | 97.77±0.84 | 97.83±0.16 |

### 8.4.6.2 Transchelation Study (DTPA Challenge)

DTPA challenge study was performed to evaluate the binding strength (transchelation) of $^{99m}$Tc with VLB. Results obtained demonstrated that presence of DTPA did not affect the labelling

efficiency. As seen from Figure 8.17, at highest concentration of DTPA i.e. 7.5 mM transchelation confirmed by ITLC was found out to be less than 3%, ensuring the stability of $^{99m}$Tc-VLB complex.

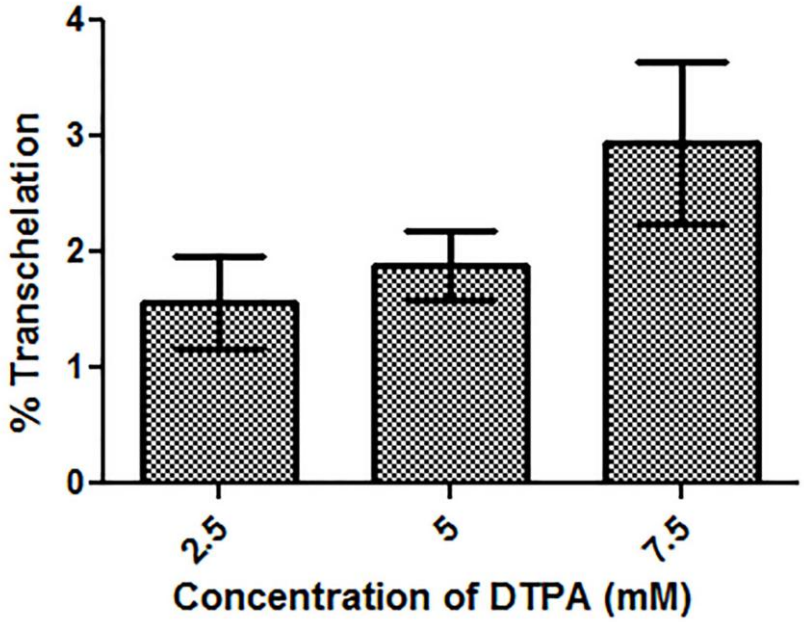


**Figure 8.17:** $^{99m}$Tc-VLB complex DTPA challenge study (n=3; mean±SD).

**8.4.6.3 *In vivo* Biodistribution studies in Rats**

Drug targeting therapies have evolved with the in depth knowledge of molecular and cellular biology of cancer and parallel improvement in innovative technology in formulation aspect. Present investigation focuses on evaluation of targeting efficiency of $^{99m}$Tc- labeled VLB loaded non-PEGylated and PEGylated Spherulites for their potential to reach non-small cell lung cancer. Sprague-Dawley rats were used to study the biodistribution of both the formulations along with $^{99m}$Tc-labeled VLB plain drug. Rats were injected a calculated amount of dose through tail vein.

Figure 8.18 and 8.19, shows that $^{99m}$Tc- labeled VLB plain drug distributed nonspecifically throughout the body of rat and high concentrations can be seen in highly perfused organs like Liver, Lungs and Kidneys. High amount of plain VLB gets metabolized mainly in liver followed by its renal clearance. Moreover, it gets bound to plasma constituents. Gamma scintigraphy images and organ distribution graph shows results in accordance with the above stated metabolism route, as high concentration of plain drug can be seen in Liver and Kidneys. High degree of metabolism and nonspecific distribution of plain VLB discourages localization of drug at targeted site i.e. Lungs. While, $^{99m}$Tc- labeled VLB loaded non-PEGylated and PEGylated Spherulites are seen to remain in circulation for longer period of time, because of

the characteristics possessed by spherulites vesicular system, as showed in Figure 8.20 (Non PEGylated spherulites) and Figure 8.22 (PEGylated spherulites). Figure 8.21 and 8.23 represents the biodistribution of Non PEGylated and PEGylated spherulites respectively in highly perfused organs. Furthermore, spherulites showed localization in Lungs, significantly more than plain drug and it was detectable for a longer period of time. As seen in Figures 8.18, 8.20, and 8.22, after an intravenous administration biodistribution within 1 hour showed that PEGylated Spherulites reach Lungs at (18.48%) which was significantly ($p<0.001$) higher concentration than non-PEGylated formulation (10.90%) followed by $^{99m}$Tc- labeled VLB plain drug (14.23%). PEGylated spherulites (14.73%) showed higher accumulation in the lungs even after 4 hours, whereas, non-PEGylated formulation and plain VLB solution were found to be 3.83% and 7.36% respectively, as seen in Figure 8.24. Gamma quanta of free drug showed its distribution throughout the body and major accumulation was seen in Liver and Kidneys, as, free drug is taken up by RES leading to the metabolism. Whereas, drug encapsulated in spherulites gets protected by the lipidic bilayers and showed stabilized circulation. Furthermore, vesicles circulation time was increased due to the PEG chains present on their surface, enabling steric stabilization of spherulites and also avoid opsonization by plasma components. PEG possesses flexible chain structure which occupies the adjacent space present on vesicles. VLB is known to have high binding with platelets and lymphocytes. Hydrophilic nature of PEG chains protects the surface of spherulites from other macromolecules by preventing the entry and binding of plasma proteins, often called as opsonins, resulting into reduced exposure of vesicles to macrophages.

Phagocytic uptake was reduced due to long-circulating spherulites which facilitated passive accumulation into targeted tissues or organs. This phenomenon is termed as passive targeting. Solid tumors tend to grow a network of capillaries i.e. angiogenesis: newly formed tumor vasculature have leaky epithelial lining, which is advantageous as vesicular formulation can easily permeate inside from systemic circulation and accumulate at the target site. Furthermore, tumor microenvironment possesses insufficient lymphatic drainage, enabling formulation to reside in the tumor vasculature for extended period of time and serve for sustained drug-delivery. This phenomenon is referred as Enhanced permeation and retention (EPR) effect. Free drug either gets metabolized or binds with plasma components making it inactive, however, efficacy of an encapsulated anticancer drug considerably increases because of the EPR mechanism. Various components can be used to PEGylate the spherulites surface. However, the most suitable method is using a block copolymer lipid like PEG-distearoylphosphatidylethanolamine (DSPE). Anchoring PEG chains onto the surface of

spherulites forms a hydrophilic layer around the vesicular membrane. Localization of PEG branches over the surface of vesicles avoids the opsonins, which, subsequently reduce the clearance or elimination of spherulites by RES. PEGylated vesicles are found to have significant increase in blood circulation time ($t_{1/2}$>40 h) along with uniform biodistribution in the tissues or organs. Targeted vesicular systems gets distributed in the blood i.e. central compartment, with minimal of only 10% to 15% of RES uptake. Conventional i.e. non-PEGylated vesicles are readily identified by RES and taken up by liver (about 80% to 90%), this indicates substantial improvement by PEG-vesicles over conventional vesicles. Vesicle surface can be attached with PEG chains by some methods as: adsorption of PEG polymer on top of the vesicle surface, formulating vesicles with attached covalent reactive groups over their surface or inclusion of PEG block copolymer in preparation method of formulation. PEG concentration is also needed to be considered as less amount can result in high RES uptake, subsequently, high concentration alters the morphology of vesicles.

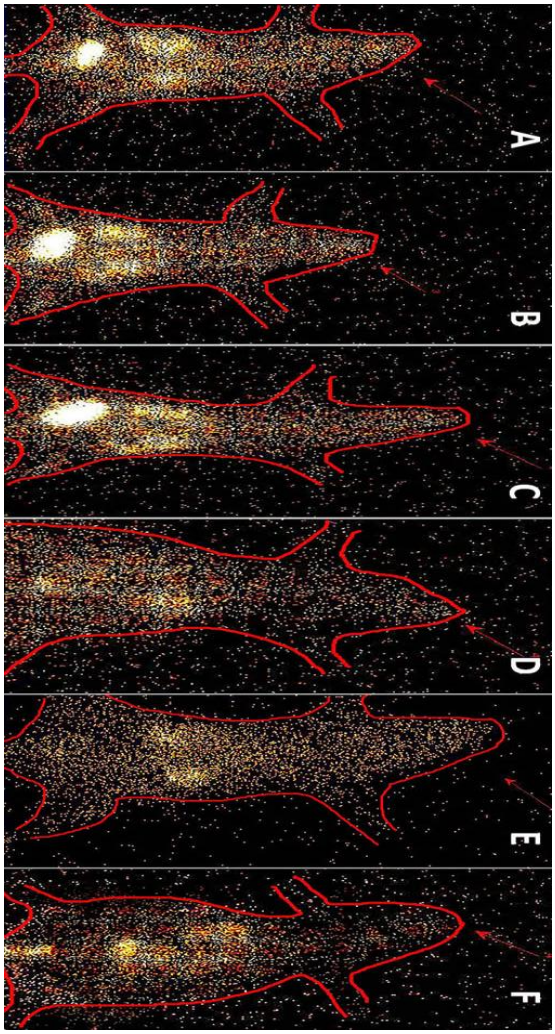


**Figure 8.18** In vivo biodistribution of $^{99m}$Tc labelled VLB plain drug solution injected intravenously in Sprague-Dawley rats, visualized by Gamma scintigraphy at different time points viz. A) 0.25 hr, B) 0.5 hr, C) 1 hr, D) 2 hrs, E) 3 hrs, F) 4 hrs.

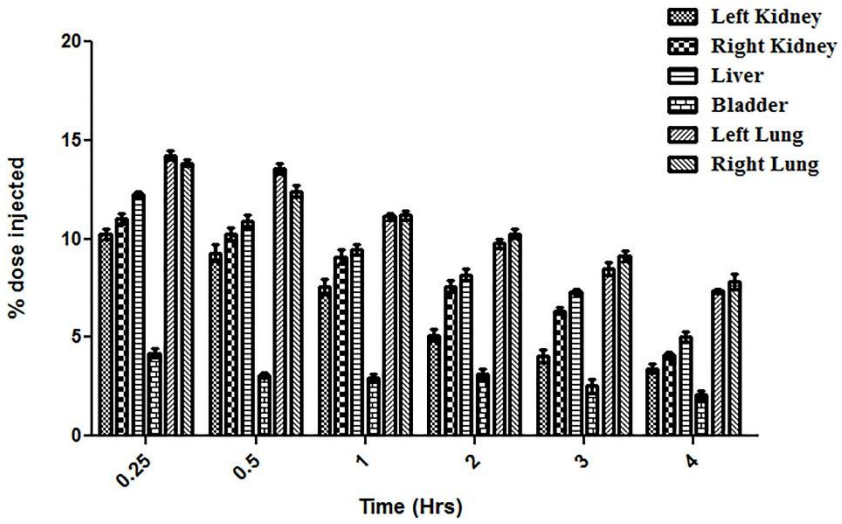


**Figure 8.19:** % dose injected intravenously, of $^{99m}$Tc labelled VLB plain drug solution in Sprague-Dawley rats (n=3), where data depicts concentration found in highly perfused organs with respect to time. Data represents mean±SD.

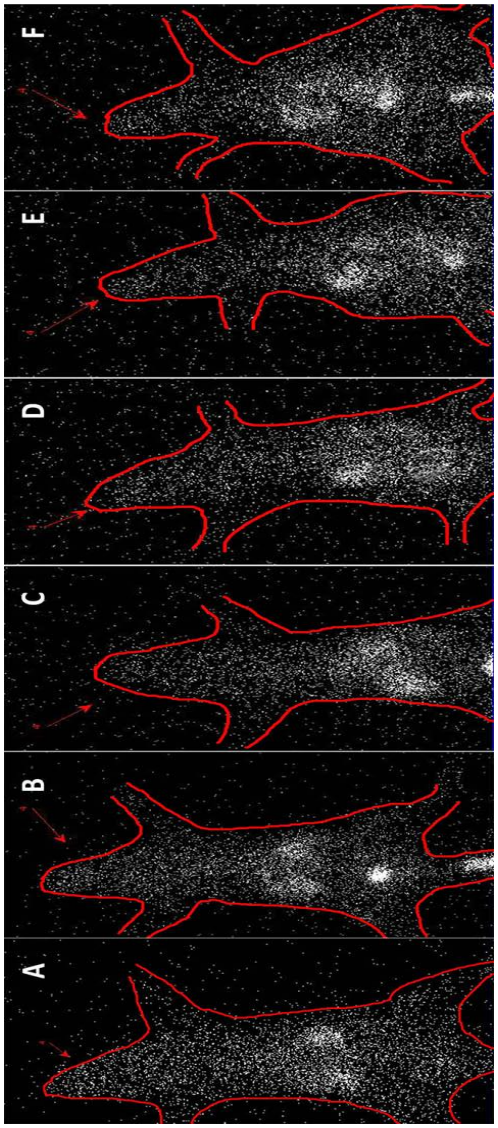


**Figure 8.20:** In vivo biodistribution of $^{99m}$Tc labelled VLB loaded Non-PEGylated Spherulites injected intravenously in Sprague-Dawley rats, visualized by Gamma scintigraphy at different time points viz. A) 0.25 hr, B) 0.5 hr, C) 1 hr, D) 2 hrs, E) 3 hrs, F) 4 hrs.

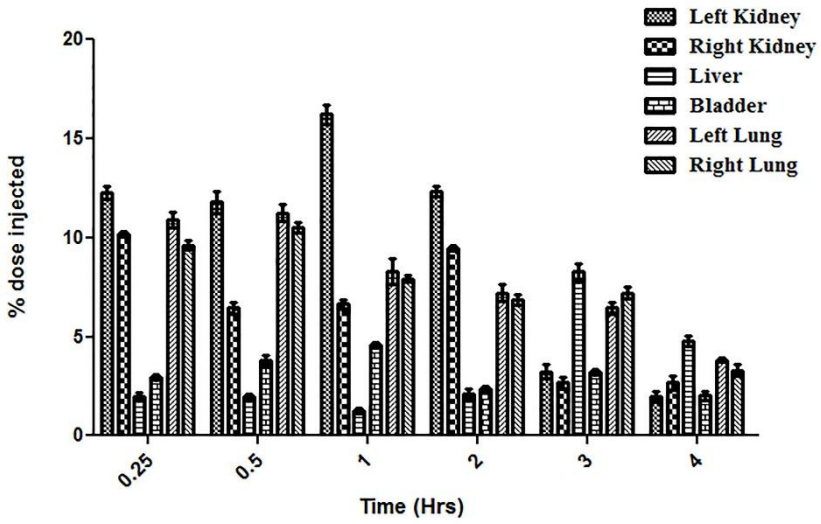


**Figure 8.21:** % dose injected intravenously, of $^{99m}$Tc labelled VLB loaded Non-PEGylated Spherulites in Sprague-Dawley rats (n=3), where data depicts concentration found in highly perfused organs with respect to time. Data represents mean±SD.

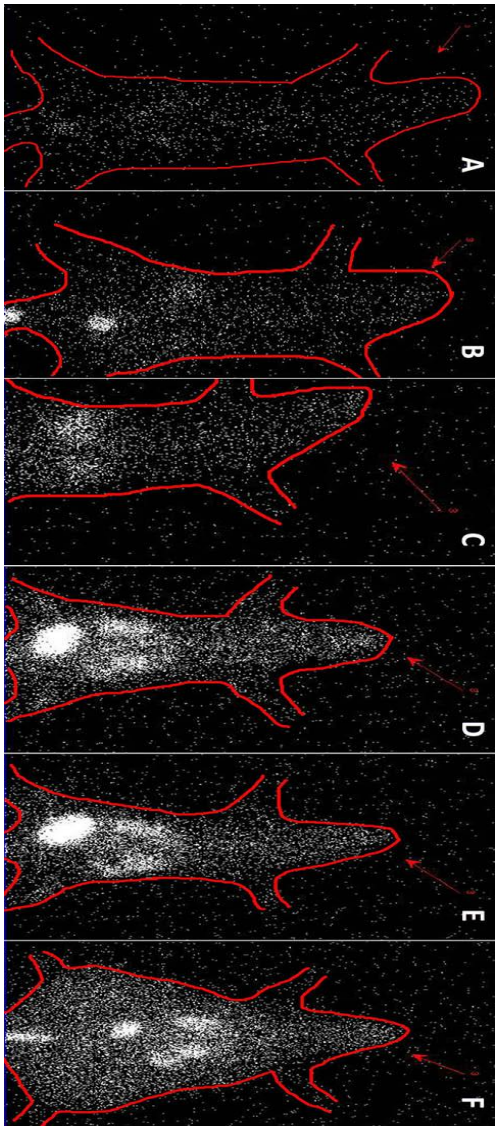


**Figure 8.22:** In vivo biodistribution of $^{99m}$Tc labelled VLB loaded PEGylated Spherulites injected intravenously in Sprague-Dawley rats, visualized by Gamma scintigraphy at different time points viz. A) 0.25 hr, B) 0.5 hr, C) 1 hr, D) 2 hrs, E) 3 hrs, F) 4 hrs.

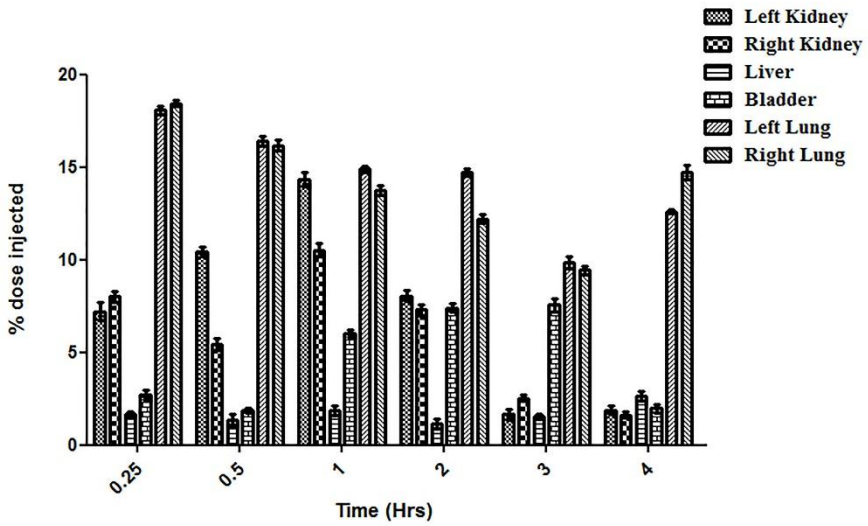


**Figure 8.23:** % dose injected intravenously, of $^{99m}$Tc labelled VLB loaded PEGylated Spherulites in Sprague-Dawley rats (n=3), where data depicts concentration found in highly perfused organs with respect to time. Data represents mean±SD.

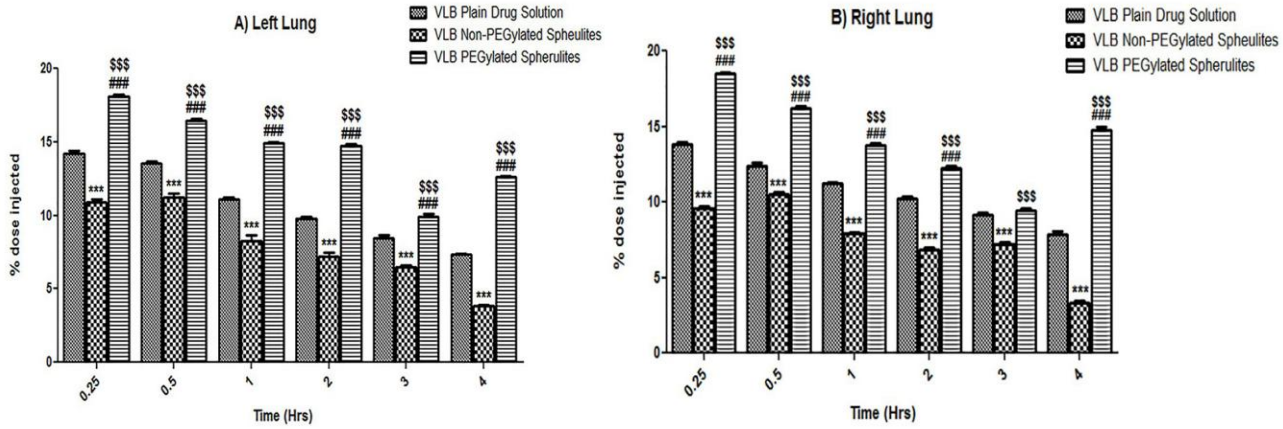


**Figure 8.24:** Lung targeting potential of VLB plain drug, VLB loaded Non-PEgylated spherulites and VLB loaded PEGylated spherulites at different time points, where: A) Left Lung, B) Right Lung. (Where, For Plain drug Solution Vs. Non-PEGylated Spherulites; *** $p<0.001$. For Plain drug Solution Vs. PEGylated Spherulites; ### $p<0.001$. For Non-PEGylated Spherulites Vs. PEGylated Spherulites; $$$ $p<0.001$) statiscally analyzed by repeated measure of Two way ANOVA followed by Bonferroni Post-tests.

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