Chapter 9
In Vivo studies

9.1 Introduction

9.1.1 *In vivo* pharmacokinetic study

Although *in vitro* techniques have reduced the numbers of animal studies still *in vivo* studies contribute greatly to understand the pharmacology, toxicology and efficacy of drugs and formulations in development. Finding the ADME (absorption, distribution, metabolism and elimination) parameters of drugs and formulations is important to avoid the failure in clinical trials. Combining *in vitro* data with *in vivo* data provides the most complete portrait about behavior of the formulation. The pharmacological effect of drug directly correlates with the concentration of the drug required, which is related to the drug concentration in blood/plasma. Therefore, the knowledge of effective blood/plasma concentration of the drug in animals can serve as a useful guide in human clinical trials. Rodents are typically used for obtaining preliminary pharmacokinetic data.

Pharmacokinetic modelling can be performed by non-compartmental or compartmental methods. Non-compartmental methods estimate the exposure to a drug by estimating the area under curve of a concentration-time graph whereas the compartmental methods estimate the concentration-time graph using kinetic models. Non-compartmental methods are often more versatile in that they do not assume any specific compartmental model and produce accurate results also acceptable for bioequivalence studies. The final outcome of the transformations that a drug undergoes in an organism and the rules that determine this fate depend on a number of interrelated factors.

The area under the plasma concentration-time curves from time zero to time infinity (AUC) was calculated by the trapezoidal rule-extrapolation method (1); this method employs the logarithmic trapezoidal rule, recommended by Chiou (2) for the calculation of area during the declining plasma-level phase, and the linear trapezoidal rule for the rising plasma-level phase. The area from the last data point to infinity was estimated by dividing the last concentration by the apparent terminal rate constant. Standard methods (1, 3) were used to calculate time-averaged total body clearance (CL), area under the first moment of plasma concentration-time curve (AUMC), mean residence time (MRT) and half-life.

9.1.2 *In-vivo* acute toxicity study

An *in-vivo* acute toxicity study in animal is essential part of drug and formulation development process. It involves precise determination of median lethal dose (LD_{50}) i.e. dose of drug required to kill the half of the treated population. The main purpose of LD_{50} determination in pharmaceutical field is that it is a practical and rapid method for comparative study of different drug substances as well as new drug products with established ones. Many methods have been developed to decrease the number of animals used in such toxicity studies and number of methods are available to have an insight about the acute toxicity of any chemical or drug product. Two methods are available now as alternatives which reduces the use of animals i.e. Fixed dose procedure (FDP) and Up-down procedure (UDP). Both methods produce data consistent with classical LD_{50} methods. Among these methods UDP requires the least number of animals (6-10) and provides results in terms of LD_{50} along with data for the hazard classification system, unlike FDP that do not estimate results in terms of LD_{50}.

For determination of LD_{50} of DTX-HSA-NPs and VBT-HSA-NPs the UDP procedure of organization for economic cooperation and development (OECD) was used. Typical protocol includes administration of a dose, a step below the level of the best estimate of the LD_{50}. If the animal dies at this dose, the next dose which is lower than the first is given to second animal; if it survives; next animal is to be given a higher dose and so on until the study has reached the precise LD_{50} value.

9.1.3 Phlebitis studies

Like other vinca alkaloids, vinorelbine is a vesicant that can cause extravasation injuries as well as local effects including phlebitis. Injection-site reactions including transient local pain, swelling and erythema. The purpose of this study was to examine the usefulness of albumin in preventing venous irritation produced by vinorelbine.

9.2 Animals

9.2.1 Pharmacokinetic study

Female Wistar rats weighing 220 ± 20 g were used for pharmacokinetics studies. Animals were housed in polypropylene cages (38cm × 23cm × 10cm) under laboratory conditions of controlled temperature 25 ± 2°C. Three rats per cage were fed ad libitum with animal feed allowing free access to drinking water. The animals were

exposed to alternate cycles of 12 hours light and darkness. All experimental procedures were reviewed and approved by the Animal Ethics Committee of Department of Pharmacy, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. All animal experiments were approved by 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.

9.2.2 Acute toxicity study

Female Swiss Albino mice (Wistar Strain) weighing 25-30 g were used for acute toxicity studies. Animals were housed in propylene cages (38cm × 23cm × 10cm) under laboratory conditions of controlled temperature 25 ± 2°C. The animals were exposed to alternate cycles of 12 hours light and darkness and fed ad libitum with animal feed allowing free access to drinking water. All experimental procedures were reviewed and approved by the Animal Ethics Committee of Department of Pharmacy, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India. All animal experiments were approved by 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.

9.3 Method

9.3.1 *In vivo* pharmacokinetic studies for DTX-HSA-NPs and VBT-HSA-NPs

Pharmacokinetic studies were performed to generate the relationship between drug dosage regimens and the concentration time profiles. DTX-HSA-NPs and Taxotere were intravenously injected at 10 mg/kg (0.5 mL) dose in rats (n=5) and VBT-HSA-NPs and Navelbine were intravenously injected at 5 mg/kg (0.5 mL) dose in rats (n=5). At the time points of 0.25, 0.50, 1, 2, 4, 6, 8, 12, 24, 48 h, 0.5 mL of blood was collected in tubes containing 0.05 ml of heparin anticoagulant (100 IU) from retro-orbital sinus under anesthesia and plasma was separated by centrifugation at 2500 rpm for 10 min. Plasma was kept frozen at –20 °C until analysis. AUC, MRT, Cl and t1/2β of DTX-HSA-NPs and VBT-HSA-NPs were determined (4, 5).

9.3.2 *In vivo* acute toxicity studies

Mice were divided in 6 groups (3 mice per group) each for Taxotere, DTX-HSA-NPs, DTX-HSA-INPs, Navelbine, VBT-HSA-NPs and VBT-HSA-INPs. Mice

were kept in their cages for at least 5 days prior to dosing to allow for acclimatization to the laboratory conditions and marked to permit individual identification. Test substances (DTX-HSA-NPs, DTX-HSA-INPs, VBT-HSA-NPs and VBT-HSA-INPs) and standards (Taxotere® and Navelbine®) were administered in a constant volume of 0.2 mL by varying the concentration of the dosing preparation (100, 125, 155, 175, 200 and 275 mg/kg for DTX and (20, 30, 37, 50, 60 and 75 mg/kg). All doses were prepared prior to administration. DTX-HSA-NPs and VBT-HSA-NPs were prepared and diluted with 0.9% normal saline to get desired dose for administration. All the test substances were sterilized by filtering through 0.2 µ membrane filter prior to administration. Prior to dosing, all the animals were fasted by withholding food but not water for 3-4 hours. The fasted body weight of each animal is determined and the dose was calculated according to the body weight. The test substances were administered via tail vein of animals using sterile single use disposable polystyrene syringes. The mice were observed for 2 weeks in all groups and the number of mice surviving was recorded and the median lethal dose (LD_{50}) was determined. After the administration of formulations, mice were withheld from food for 1-2 h except for the case where dosing is done in fractions.

9.3.3 VBT induced phlebitis studies in rats

Studies were performed on Wistar albino rats (250-300 g). Animals were anesthetized by intraperitoneal injection of ketamine (100 mg/kg). After confirming anesthesia (through pinching) rats were laid on a surgical table such that the ventral surface of the body faced upwards. During the entire procedure temperature was maintained with the help of a 60 watt lamp positioned on top of the animal. A minor incision was placed in the inner thigh region of the right hind paw and connective tissue was seared using pointed forceps to expose the right femoral vein. A 28-gauge needle connected to a PE catheter was inserted in the lower region of the vein for administration of test solutions. Test solutions were injected at (volume 0.2 ml) time 0 and development of inflammation/ edema of the vein were observed for a period of 30 minutes. The left counter paw was utilized as control to confirm inflammation in the test paw. At the end of the procedure the animals were rehabilitated to the animal house as deemed appropriate. If required, animals were treated with commercially available diclofenac gel on the affected areas, before returning them to animal house.

9.4 Statistical Analysis

Data were expressed in mean ± SEM. Statistical analysis was performed by analysis of variance (ANOVA) and Student's t-test and differences at $P<0.05$ were considered significant.

9.5 Result and Discussion

9.5.1 *In vivo* pharmacokinetic studies of DTX-HSA-NPs

Validated RP-HPLC method was used to determine plasma concentration of DTX. DTX- HSA-NPs and Taxotere were intravenously injected at 10 mg/kg (0.5 mL) dose in rats (n=5) and the blood was collected for pharmacokinetic evaluation and analyzed by RP-HPLC. Non-compartmental analysis of the plasma concentrations showed a significant change in pharmacokinetic parameters of DTX in HSA-NPs compared to that of commercial formulation. The Pharmacokinetics parameters of the two formulations were shown in **Table 9.1** and plasma concentration-time graph shown in **Figure 9.1.** DTX-HSA-NPs showed significantly higher AUC, MRT and $t_{1/2}$ (16.890 ± 1.541 µg. h/mL, 6.651 ± 0.65 h and 7.452 ± 1.24 h) compared to Taxotere® (8.796 ± 1.121 µg. h/mL, 2.491 ± 0.46 h and 2.693 ± 1.65 h) whereas The DTX-HSA-NPs also showed significantly decreased clearance (592.2±15.1 mL/h) compared to Taxotere® (1136.9±23.5 mL/h). The results of *in vivo* pharmacokinetic studies clearly revealed better pharmacokinetic properties of DTX-HSA-NPs compared to marketed formulation of DTX (Taxotere®).

Table 9. 1 *In vivo* pharmacokinetic parameters of DTX in DTX- HSA-NPs and Taxotere after i.v. injection in SD rats (10 mg DTX/kg) (n = 5)

Formulation	*AUC (µg. h/mL)	MRT (h)	Cl (mL/h)	T1/2β (h)
DTX-HSA-NPs	16.890±1.541	6.651±0.65	592.2±15.1	7.452±1.24
Taxotere	8.796±1.121	2.491±0.46	1136.9±23.5	2.693±1.65

*$AUC_{0-\infty}$: Area under the plasma concentration time curves;

MRT: Mean retention time;

Cl: Clearance

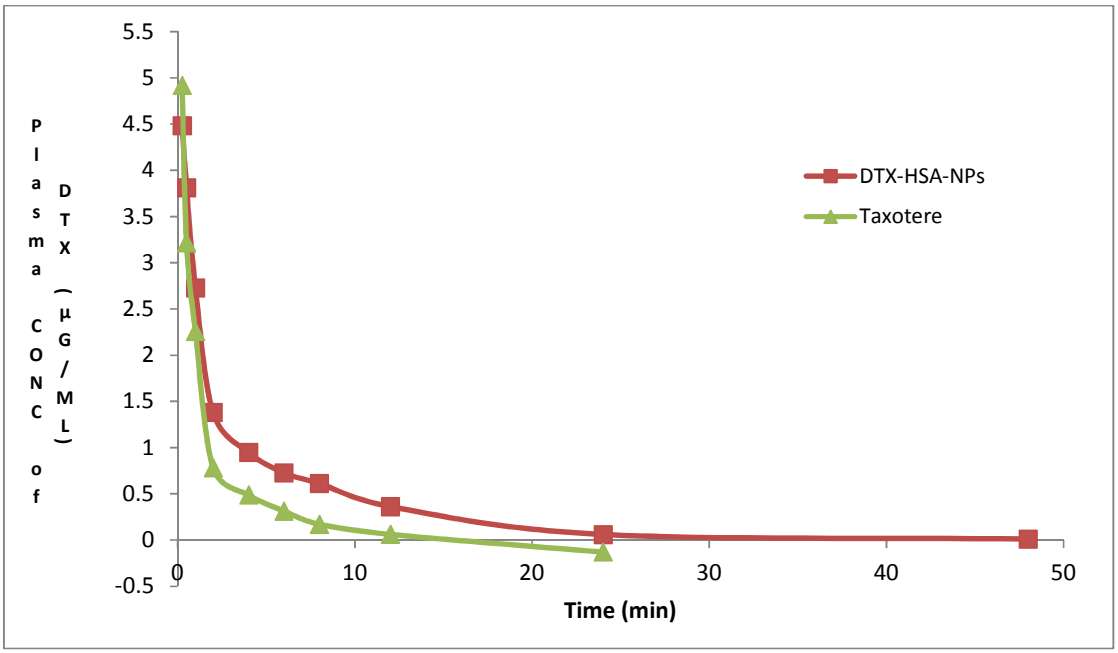

Figure 9. 1 Mean plasma concentration–time profiles of DTX following IV injection of two different formulations of DTX at a dose of 10 mg/kg in rats.

9.5.2 *In vivo* pharmacokinetic studies for VBT-HSA-NPs

Validated RP-HPLC method was used to determine plasma concentration of VBT. VBT-HSA-NPs and Navelbine were intravenously injected at 5 mg/kg dose (0.5 mL) and the blood was collected for pharmacokinetic evaluation and analyzed by RP-HPLC. The non-compartmental analysis of the plasma concentrations showed a significant change in pharmacokinetic parameters of VBT in HSA-NPs compared to that of commercial formulation. Pharmacokinetics parameters of the two formulations were shown in **Table 9.2.**

VBT-HSA-NPs showed significantly higher AUC, MRT and $t_{1/2}$ (40.03 ± 1.28 µg. h/mL, 33.25 ± 1.67 h and 33.34 ± 1.54 h) compared to Navelbine® (7.314 ± 2.13 µg. h/mL, 3.407 ± 0.85 h and 3.926 ± 1.35 h) whereas the VBT-HSA-NPs also showed significantly decreased clearance (124.90 ± 11.62 mL/h) compared to Navelbine® (683.59 ± 23.41 mL/h). The results of *in vivo* pharmacokinetic studies clearly showed better pharmacokinetic properties of VBT-HSA-NPs compared to marketed formulation of VBT (Navelbine®).

Table 9. 2 *In vivo* pharmacokinetic parameters of VBT in VBT-HSA-NPs and Navelbine after i.v. injection in SD rats (5 mg VBT/kg) (n = 5)

Formulation	*AUC (µg. h/mL)	MRT (h)	Cl (mL/h)	T1/2β (h)
VBT-HSA-NPs	40.03 ± 1.28	33.25 ± 1.67	124.90±11.62	33.34 ± 1.54
Navelbine	7.314 ± 2.13	3.407 ± 0.85	683.59±23.41	3.926 ± 1.35

*$AUC_{0-\infty}$: Area under the plasma concentration- timecurves;

MRT: Mean retention time;

Cl: Clearance

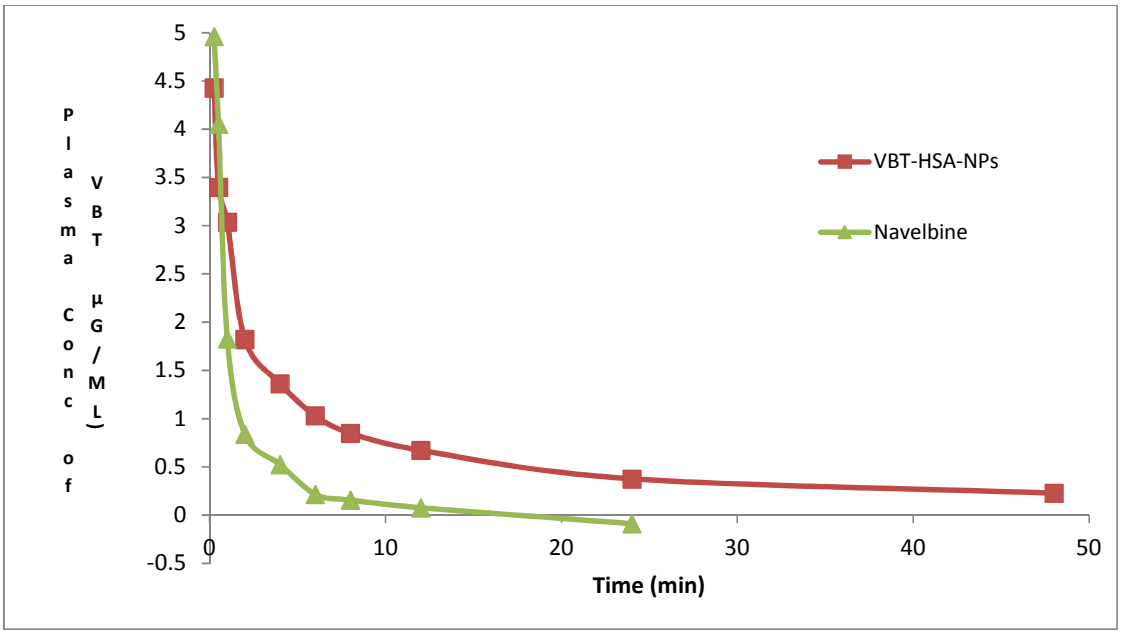

Figure 9. 2 Mean plasma concentration time profiles of VBT following IV injection of two different formulations of VBT at a dose of 5 mg/kg in rats.

9.5.3 *In vivo* acute toxicity studies of DTX

LD_{50} was determined by observing the signs of morbidity and mortality in the mice after dosage administration of all three formulations. LD_{50} of Taxotere was at 155 mg/kg which was concordant to previously estimated LD_{50} (>154 mg/kg) in mice via i.v. route (Taxotere Label Information). The LD_{50} of DTX-HSA-NPs and DTX-HSA-INPs was about 200 mg/kg which is better than that of marketed formulation. Thus the toxicity of marketed formulation can be reduced by encapsulating the DTX in nanoparticulate system that can provide better alternative for administration of DTX.

9.5.4 *In vivo* acute toxicity studies of VBT

LD50 was determined by observing the signs of morbidity and mortality in the mice after dosage administration of all three formulations. LD_{50} of Navelbine was roughly 37 mg/kg which was concordant to previously estimated LD_{50} (36.1 mg/kg) in mice via i.v. route (Navelbine Label Information). The LD_{50} of VBT-HSA-NPs and VBT-HSA-INPs was about 60 mg/kg which is better than that of marketed formulation. Thus the toxicity of marketed formulation can be reduced by encapsulating the drug in nanoparticulate system providing a better alternative for administration of VBT.

9.5.5 VBT induced phlebitis studies in rats

Major sign of inflammation/edema of the vein was observed in Plain VBT solution **(Figure 9.3)** whereas minor sign of inflammation/edema of the vein was observed in albumin + VBT Physical mixture **(Figure 9.4)**, whereas no sign of inflammation/edema of the vein was observed VBT-HSA-NPs **(Figure 9.5)** and VBT-HSA-INPs **(Figure 9.6)** which indicates that development of VBT loaded HSA-NPs prevents development of inflammation/edema of the vein in rats may be due entrapment of drug inside albumin nanoparticles.

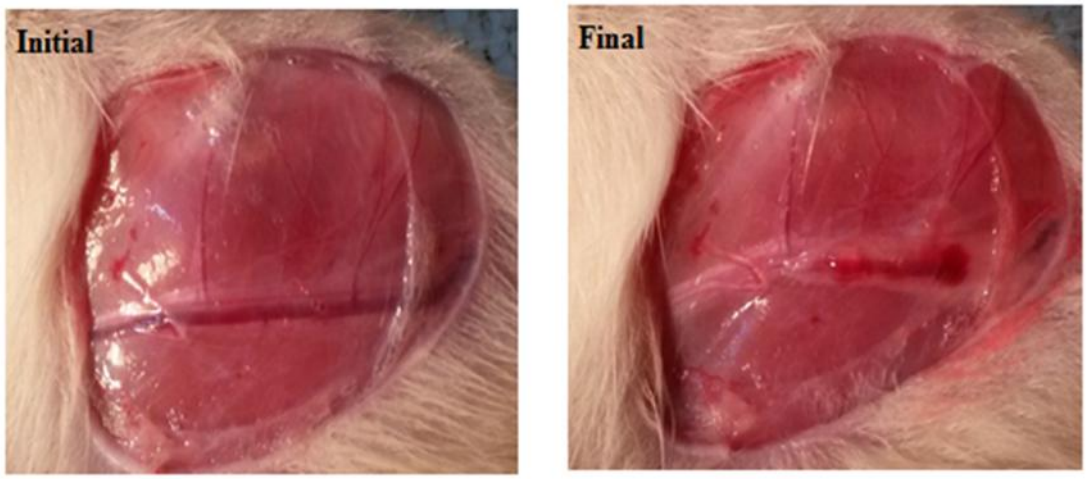

Figure 9. 3 Plain VBT Solution

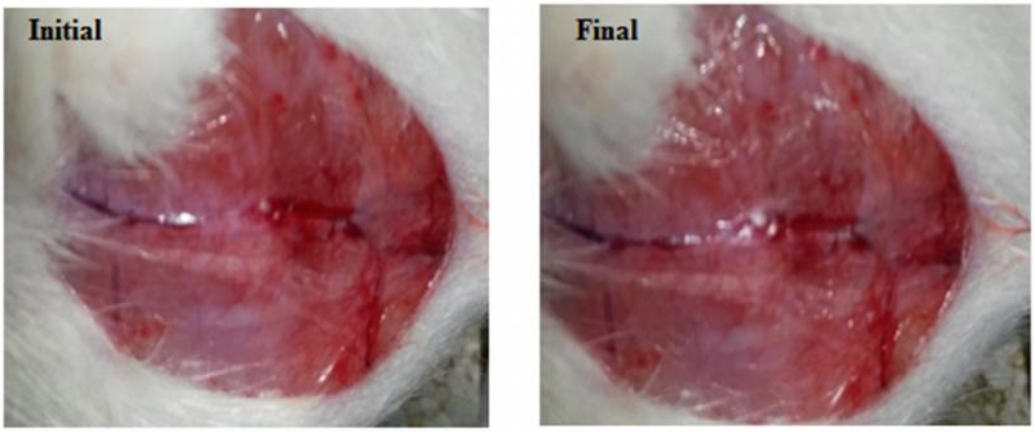

Figure 9. 4 Albumin+ VBT Physical mixtures

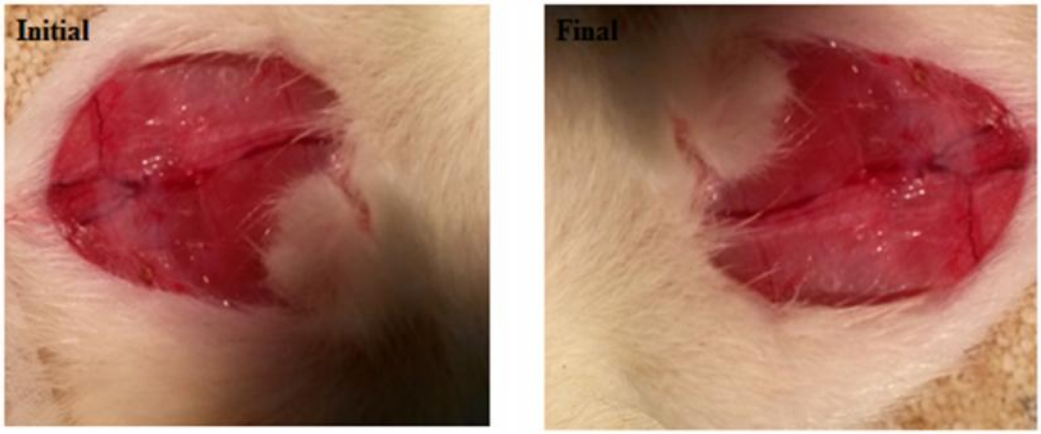

Figure 9. 5 VBT-HSA-NPs

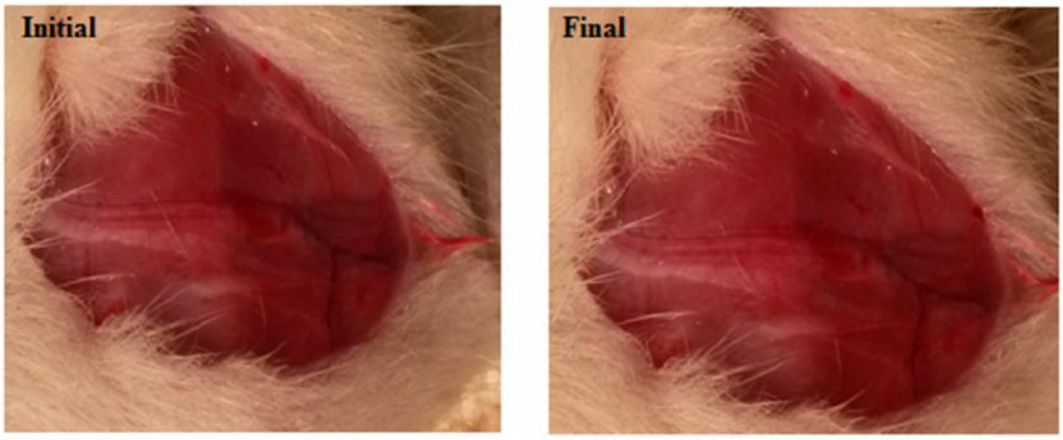

Figure 9. 6 VBT-HSA-INPs

9.6 References

1. Chen ML, Chiou WL. Tissue metabolism and distribution of methotrexate in rabbits. Drug Metab Disp. 1982;10:706-7.
2. Chiou WL. Critical evaluations of potential error in pharmacokinetic studies using the linear trapezoidal rule method for the calculation of the area under the plasma level-time curve. J Pharmacokinet Biopharm. 1978;6:539-46.
3. Riegelman S, Collier P. The application of statistical moment theory to the evaluation of in vivo dissolution time and absorption time. J Pharmacokinet Biopharm. 1980 (8):509-34.
4. Song H, Geng H, Ruan J, Wang K, Bao C, Wang J, et al. Development of Polysorbate 80/Phospholipid mixed micellar formation for docetaxel and assessment of its in vivo distribution in animal models. Nanoscale research letters. 2011;6(1):354. PubMed PMID: 21711889. Pubmed Central PMCID: PMC3211444. Epub 2011/06/30. eng.
5. Su M, Zhao M, Luo Y, Lin X, Xu L, He H, et al. Pharmacokinetics and tissue distribution of vinorelbine delivered in parenteral lipid emulsion. European Journal of Lipid Science and Technology. 2011;113(2):152-9.