Chapter-8
In vivo studies

8.1 Introduction

8.1.1 Hemolysis Study

Liposomes due to their similarity with biological membranes are biocompatible and principally nontoxic to blood cells. However occasionally, hemolytic activity is seen with the liposomal components (1, 2). It has been also documented that several lipids containing short-chain phosphatidylcholines and lipid metabolites like free fatty acids and lysophosphatidylcholine induce erythrocyte lysis by some non-specific destruction of cell wall causing various Sodium (Na+) and potassium (K+) ion permeability defects (2, 3). This might be related to the hemolytic activity of various liposomes and lipid component.

Phospholipids are susceptible to undergo various physicochemical changes on exposure to different conditions (4, 5). Chemically phospholipids are vulnerable to hydrolytic reactions at the ester bonds. Hydrolysis induces formation of lysophosphatidylcholine and free fatty acids (6) and causes increase in membrane permeability (7). Such changes also encourage changes in organization of liposomes initiating transformation of liposomes to micellar solutions (7). Such components as listed above can cause erythrocyte lysis by getting incorporated in erythrocyte membrane and causing ion permeability defects. This necessitates the evaluation of hemolysis potential of liposomes. Hemolytic toxicity of formulated liposomes was checked by incubating the formulation with Red Blood Cells separated from Rat blood by centrifugation at low speed (8) and analysing the samples for hemoglobin release at 540 nm (1). The hemolysis with different formulations was compared with that obtained with Triton-X100 as a positive control (9).

8.1.2 Pharmacokinetic study

Although *in vitro* methods give an idea about the evaluation of test sample or formulation without using animal and simulate the conditions of *in vivo* studies, *in vivo* studies contribute importantly to know and 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 essential to evade the failure in clinical trials. Combining *in vitro* data with *in vivo* data provides the most comprehensive representation about behaviour of the formulation and

drugs. 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 data and understanding of effective blood/plasma concentration of the drug in animals can serve as a beneficial 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 that are 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 (10); this method employs the logarithmic trapezoidal rule, recommended by Chiou (11) 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 (10, 12) 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.

8.1.3 Acute Toxicity Study

In vivo acute toxicity studies on animals are an essential part of drug development process. Such acute toxicity studies are carried out for various objectives i.e.

1. To determine the Median Lethal Dose (LD_{50}) after a single dose administered through one or more routes, one of which is the intended route of administration in humans.

2. To determine Maximum Tolerated Dose (MTD) and No Observable Effect Level (NOEL).
3. To identify potential target organs for toxicity, determine reversibility of toxicity, and identify parameters for clinical monitoring.
4. To help select doses for repeated-dose toxicity tests.

A number of methods are available to have an insight about the acute toxicity of any chemical or drug product. These include classical Litchfield and Wilcoxon method (Dosing of animals of both sex with increasing amounts of chemical and plotting dose-response curve to determine LD_{50}/MTD). This type of study has a disadvantage that it uses a large number of animals. So two methods are available now as alternatives which reduces the use of animals i.e. Fixed Dose Procedure (FDP) (13) and Up-Down Procedure (UDP) (14). Both methods produce data consistent with classical LD_{50} methods (15, 16). Among these methods Up-Down procedure requires the least number of animals (6-10) of single sex and provides results in terms of LD_{50} along with data for the hazard classification system, unlike FDP that does not estimate results in terms of LD_{50} value (17). Instead FDP gives better evaluation of the maximum tolerated dose of drug/drug product.

MTD of a drug can be defined as the highest dose of a drug or treatment that does not cause unacceptable side effects. The maximum tolerated dose is determined in clinical trials by testing increasing doses on different groups of people until the highest dose with acceptable side effects is found. Toxicity parameters to be considered include,

1. Mortality
2. Clinical pathology
3. Gross necropsy
4. Weight change
5. Signs of toxicity – convulsions, rashes, akinesia, licking, tremors

Drug doses at or below this level should not induce (18)

- Overt toxicity, for example appreciable death of cells or organ dysfunction,
- Toxic manifestations that are predicted materially to reduce the life span of the animals except as the result of neoplastic development or

- 10% or greater retardation of body weight gain as compared with control animals.

8.2 Materials and Instruments

Materials:

Sr No	Chemicals/Materials	Source/Manufacturer
1.	Triton X-100	Himedia lab. Pvt. Ltd., India
2.	Glacial Acetic Acid (HPLC)	Spectrochem, India
3.	Acetonitrile (HPLC)	Spectrochem, India
4.	Methanol (HPLC)	Spectrochem, India

Instruments:

Sr No	Instruments	Company
1.	BOD Shaker Incubator	Orbitek, Scigenics
2.	Centrifuge	Remi Sci. Equipment, India
3.	UV Visible Spectrophotometer (1800)	Schimadzu, India
4.	RP-HPLC	Shimadzu LC-20AT, Japan

Animals:

Sprague Dawley (SD) female rats weighing 220 ± 20 g and Balb/c female mice weighing 25 ± 3 g were obtained from Zydus Research Center, Ahmedabad, India. All the protocols were approved by the Institutional Animal Ethics Committee. The temperature in the animal room was 20-25°C. Artificial lighting with the sequence of 12 hr light and 12 hr dark was kept in animal housing. The animals were housed individually. For feeding, conventional rodent laboratory diets was used with an unlimited supply of drinking water. All experiments and protocol described in the present study were approved by the Institutional Animal Ethical Committee (IAEC) of Pharmacy Department, The M. S. University of Baroda and with permission from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

8.3 Methods

8.3.1 Hemolysis Study

For determination of toxicity of the formulation to the red blood cells, hemolysis study was carried out as reported earlier (19). Samples of Taxol®, DLPLs and ILs were evaluated for their hemolytic potential. The samples were diluted with normal saline to 5000, 500, 50 and 5 µg/ml. Blood was collected by retroorbital puncture from rats in heparinized tubes and was centrifuged in cooling condition for 10 min at 3000 rpm. The resulting cell pellet was then reconstituted in normal saline to obtain 2 % RBC suspension. The resulting RBC suspension was then treated with 0.5 % v/v solution of triton X-100 to completely hemolyze the RBC and the absorbance values were considered as 100 %. Similarly, the absorbance values for the normal saline treated RBC suspension, that did not produce any hemolysis were taken as blank. The above dilution samples for Taxol®, DLPLs and ILs were mixed with the RBC suspension and incubated for period of 1 hr at 37±1 °C in incubator. After incubation, the samples were centrifuged at 3000 rpm for 10 min at 4 °C to sediment the non-lysed cells from the suspension. The absorbance values of the resulting supernatant were measured after appropriate dilution using PBS (pH 7.4) at wavelength of 540 nm. The relative hemolysis (in percentage) of each sample was calculated using the following formula.

$$\text{Hemolysis (\%)} = (\text{Abs sample} - \text{Abs saline})/(\text{Abs triton} - \text{Abs saline}) \times 100$$

Where Abs sample, Abs saline, and Abs triton are the absorbance of the samples (Taxol®, DLPLs and ILs), negative control (0.9% Nacl) (0% hemolysis) and 0.5% v/v Triton X 100 as positive control (for 100 % hemolysis) respectively.

8.3.2 Pharmacokinetic study

Female Sprague-Dawley rats (220 ± 20 g) were used for pharmacokinetic evaluation of prepared formulation. Rats were divided into three groups, with six in each group. All the animals were fasted overnight on the day prior to experiment. Comparison of Pharmacokinetic profile of Taxol®, DLPLs and ILs was carried out. Dilution of all the formulation was prepared using 5% dextrose solution to achieve dose

of 5 mg/kg. Formulations were injected to the tail vein of rats via single intravenous injection. At definite time interval of 0, 0.5, 1, 2, 4, 6, 8, 12, 24 h post injection, blood samples were collected from retroorbital puncture in tubes containing heparin solution. The blood samples were centrifuged at 3000 rpm for 10 min at 4°C in cooling centrifuge to separate plasma. The samples were stored at -20°C until analysis. The plasma samples were analysed for PTX content using RP-HPLC. Data were analysed using Kinetica software (Version 5.1) for non-compartment modelling to obtain various pharmacokinetic parameters.

8.3.3 Acute Toxicity Study

The maximum tolerated dose (MTD) and 50% lethal dose (LD50) for Taxol® and DLPLs were assessed in healthy female Balb/c mice for I.V. route after a single dose. Animals were randomly divided into twelve groups each containing 6 mice. The samples were diluted/reconstituted in 5% dextrose solution. The first group of animals acted as mock control and did not receive any treatment. The second group served as a control, received dextrose solution and remaining two groups received Taxol® and DLPLs diluted with 5 % dextrose. Doses were escalated in 2-fold increments starting at 6 mg/kg i.e. 6, 12, 24, 48, 96 and 192 mg/kg and Taxol® was 6, 12, 24 and 48 mg/kg. Mice were observed for up to 14 days following treatment for variation in weight, signs of toxicity, death and latency to death.

8.4 Result and Discussion

8.4.1 Hemolysis study

Hemolysis assay is a useful *in vitro* hematological test as an indicator of toxicity and physiologic compatibility of the formulation. Toxic formulations tend to cause membrane damage to the RBC leading to its rupture and release of haemoglobin. Results of the hemolytic potential of the Taxol®, DLPLs and ILs at various concentration range are presented in the Figure 8.1. It was observed that both the tested formulation exhibited concentration dependent hemolysis. At highest concentration tested, i.e. 5 mg/ml, for Taxol® hemolysis was ~48.5 %, whereas that for DLPLs and ILs was only 12.10 % and 11.59% respectively ($p<0.05$). The relative % hemolysis for both the formulations (DLPLs and ILs) at 0.5 and 5 µg/ml were approximately 4.5-fold

and 4-fold lower respectively than the corresponding tested concentration of the Taxol®. The low toxicity potential of our DLPLs and ILs system was due to use of phospholipids that mimic biological membrane and are biocompatible.

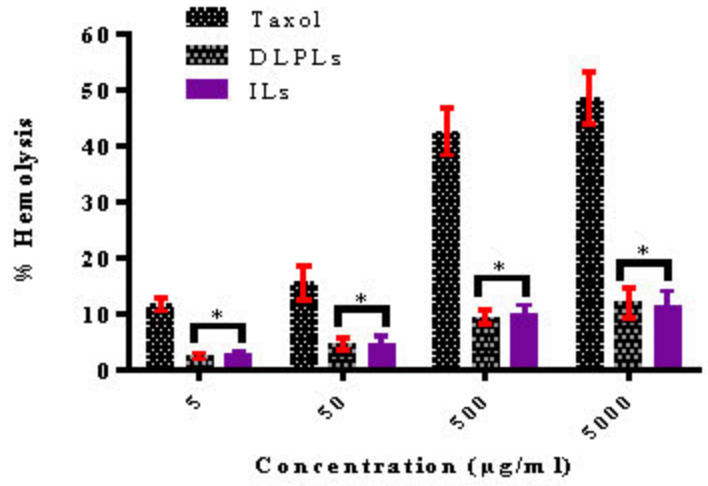


Figure 8.1: Hemolytic potential of DLPLs and ILs in comparison to Taxol® (*p<0.05)

8.4.2 Pharmacokinetic study

The plasma concentration-time profiles of PTX after single intravenous bolus administration of the PTX formulations (5 mg/kg) were characterized in Sprague Dawley rats. The main pharmacokinetic parameters of PTX are summarized in Table 8.1. As shown in Figure 8.2, PTX in Taxol® was quickly eliminated after intravenous administration with MRT and $t_{1/2}$ of 3.61 and 2.82 hr, respectively. However, PTX in DLPLs and ILs was more slowly eliminated from the circulation than Taxol®. The values of MRT and $t_{1/2}$ in the treatment groups significantly increased to 12.89 hr and 10.68 hr for DPLPs and for ILs respectively. This indicates that DLPLs and ILs were able to act as PTX reservoirs in blood giving a prolonged release profile. Consequently, the resultant values of AUC in the DLPLs and ILs treatment groups were significantly increased of the order of 4.37 and 4.10 folds compared with those in the Taxol®. Due to superior *in vitro* cytotoxicity of DLPLs and ILs as compared to PLs, only DLPLs was taken forward for *in vivo* evaluation along with ILs and PLs were not taken.

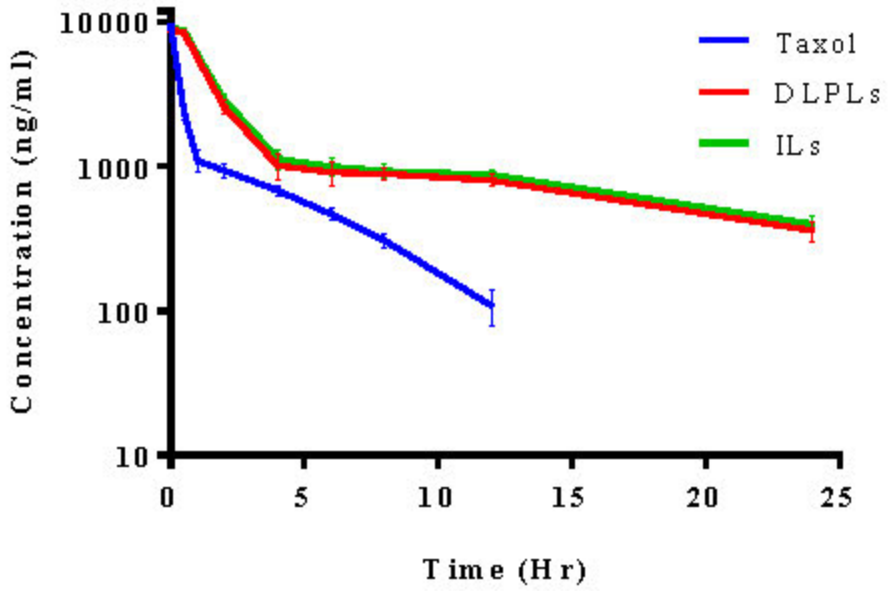
Table 8.1: The pharmacokinetic parameters of PTX after intravenous administration of Taxol®, DLPLs and ILs at 5 mg/kg PTX in SD rats (mean±S.D., n=6).

Pharmacokinetic Parameter	*Unit*	*Taxol®*	*DLPLs*	*ILs*
C_0	*μg/ml*	9.34±1.98	9.48±1.56*	10.92±1.26**
T1/2	*hr*	2.82±1.06	12.24±0.89**	12.46±0.65**
AUC	*hr.μg/ml*	9.07±1.68	39.63±4.33**	37.21±5.41**
MRT	*hr*	3.61±0.36	12.89±0.72**	10.68±0.86**
Cl	*ml.kg/hr*	551.39±88.60	126.17±15.23**	175.84±21.49**
Vss	*L/kg*	1.99±0.29	1.63±0.26**	1.69±0.39*

**p < 0.01 vs Taxol group*

***p < 0.05 vs Taxol group*

A.



B.

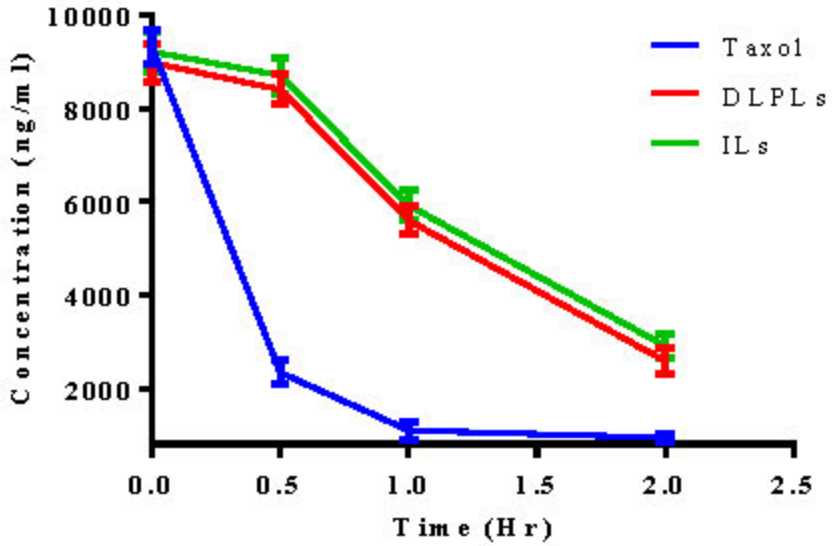


Figure 8.2: Pharmacokinetic profile of PTX after single intravenous injection of Taxol®, DLPLs and ILs in rats. (A) full profile (B) magnified profile in concentration range of 1500-1000 ng/ml. Data are presented as mean ± SD.

Both our formulation displayed superior pharmacokinetic effect that may be attributed to the longer circulation time due to PEGylation along with the higher loading of the vesicles. Further, a rapid decrease in the plasma concentration profile of Taxol® may be due to its rapid clearance from the circulation and tissue redistribution whereas for the DLPLs and ILs, after a phase of initial steep decrease due to release of PTX from the bilayer and its clearance, a subsequent gradual decrease in the concentration profile was observed. This may be correlated and is consistent with the *in vitro* release profile from DLPLs and ILs that exhibit an initial burst release followed by a phase of slow release. But, it can also be noted that an exact correlation between *in vitro* release profile and plasma concentration time profile cannot be estimated as the formulation encounters more barriers that the simple *in vitro* release test could not simulate.

8.4.3 Acute Toxicity Study

The main objective of acute toxicity study was to identify toxicity profile of DLPLs in comparison to Taxol®. The study was conducted in the dose range of 6 to 192 mg/kg and was administered by intra venous route (i.v). Tables 8.2 summarize the

result of acute toxicity of DLPLs and Taxol®. MTD for DLPLs and Taxol® was found to be 120 and 12 mg/kg, respectively. The LD50 was found to be 136.9 mg/kg and 19.7 mg/kg, respectively for DLPLs and Taxol®. Clinical signs for toxicity were observed in groups treated with higher dose of DLPLs and Taxol®. There were no treatment related mortality and toxic symptoms in animals treated with liposomal formulation at dose upto 120 mg/kg and in the Taxol® upto 12 mg/kg. All animals showed normal behavior and no treatment related clinical symptoms were shown as those in the control group. However, at the higher dose of DLPLs and Taxol® clinical signs of toxicity such as lethargy, skin pallor, rough fur and hair loss were observed. Food consumption was also found to decrease at a higher dose of treatment. Normal weight gain was observed at lower dose treatment upto 120 mg/kg and 20 mg/kg in liposomal and Taxol®, respectively. Decrease in body weight was observed at higher dose of treatment (Table 4). Thus, the results of acute toxicity study confirmed the safety of PTX-liposome formulation in healthy female BALB/c mice than Taxol® formulation after a single I.V. dose. The MTD and LD50 for DLPLs were 10-folds and 7-fold higher compared to Taxol®.

Table 8.2: Acute toxicity study results for DLPLs vs Taxol®.

Group	*Formulation (mg/kg)*	*Survival Rate*	*Mortality latency[a] (hr)*	*Toxic Symptoms*	*Behavioral changes*	*% Change in body weight after 14 days*
1	Control	6/6	No	None	None	+1.8 ±0.3
			PTX DLPLs			
2	6	6/6	No	None	None	+2.1 ±0.6
3	12	6/6	No	None	None	+2.3 ±0.2
4	24	6/6	No	None	None	+1.6 ±0.7
5	48	6/6	No	None	None	+2.0 ±0.3
6	96	6/6	No	None	None	+2.2 ±0.5
7	192	1/6	< 24	Convulsions	Lethargy	- 4.8 ±0.3
			Taxol®			
8	6	6/6	No	None	None	+1.8 ±0.3
9	12	6/6	No	None	None	+1.8 ±0.3
10	24	2/6	>24 & < 42	Convulsions	Lethargy	-5.1 ±0.6
11	48	1/6	< 24	Convulsions	Lethargy	-5.3 ±0.4

Data are presented as mean ± SD (n=6); a=Time to death in hr after injection

8.5 References

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