8. Introduction

Pharmacokinetic data interpretation can be viewed primarily as an effort to deduce what has happened to a drug in the body after administration based on its exposure to biological fluids such as plasma or blood [1]. The pharmacological effect of a drug directly correlates with the concentration of the drug in target site, 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 to human clinical trials. Rodents are typically used for obtaining preliminary pharmacokinetic data [2].

8.1.Pharmacokinetic study

Pharmacokinetic studies were performed on Female Wistar rats weighing 200-300 g for in vivo pharmacokinetic studies for SPANs (surface modified pH sensitive Fe_2O_3@FePt alloy nanoconjugates (Fe_2O_3@FePt-NH_2/COOH-Drug-HA-Ctx)) and M-SPANs (Fe_2O_3@FePt-NH_2/COOH-TPP-Drug-HA-Ctx) to obtain plasma concentration-time profile. The study protocol was approved by the Institutional Animal Ethics Committee (MSU/IAEC/2016-17/1631) of The Maharaja Sayajirao University of Baroda. The rats were housed in cages placed in an animal room with a constant temperature of 25±2°C and a fixed 12-hour light-dark cycle. All procedures were carried out in accordance with the CPCSEA guidelines, Department of Animal Welfare, Government of India. The rats were maintained on a standard diet with free access to water. After acclimatization, rats were randomly allocated to 7 groups with six animals (n=6) in each group for pharmacokinetic study as given in Table 8.1:

Table 8.1: Groups of animals for pharmacokinetic study

Groups	Administered samples
Group 1	Normal control
Group 2	Drug Suspension (Intranasal)
Group 3	Drug Suspension (Intravenous)
Group 4	SPANs (Intranasal)
Group 5	SPANs (Intravenous)
Group 6	M-SPANs (Intranasal)
Group 7	M-SPANs (Intravenous)

8.1.1. Animal dose calculation

Animal equivalent dose was calculated using following formula:

[AED] (mg/kg) = [HED] (mg/kg) * [Km ratio for Rat]

Where,

AED = Animal Equivalent Dose

HED = Human Equivalent Dose

Here, HED = 5mg/60kg = 0.083mg/kg

Km ratio for Rat = 6.2

So, AED = 0.5146mg/kg.

Average weight of animal was considered 250g. So, the dose to be administered through oral route to a single animal was calculated and found to be 0.129 mg.

The oral bioavailability of drug is ~33%. So, for parenteral route, the dose to be administered through i.v route to a single animal was found to be 0.0425 mg (considering 100% bioavailability via. parenteral route) [3].

8.1.2. Sample administration and blood collection

Pharmacokinetic studies on female Wistar rats (200-300g) were performed for plain drug suspension, SPANs and M-SPANs to obtain the plasma concentration–time profile by intranasal and intravenous route. The animals were randomly divided into 7 groups as given in table 8.1.After administration by intranasal route (using PE-10 tubing connected to micropipette (100 µL)). and intravenous route (0.0425 mg, 50µL) (in rat tail vein using 2 mL syringe with 25G needle)at predetermined intervals (1h, 2h, 4h, 12h and 24h), the blood was collected retro-orbitally (0.5 ml) from the animals in eppendorfs containing 30 µl of EDTA. Plasma was separated from the collected blood samples by centrifugation at 4000 rpm for 15 minutes. The plasma was carefully separated in eppendorfs and stored at -20 °C until further analysis.

8.1.3. Brain Targeting Efficiency

For studying deposition of drug in brain, rats (n=6) were administered SPANs (equivalent to 0.0425 mg LND, 50µL) and M-SPANS (equivalent to 0.0425 mg LND, 50µL) along with LND solution (0.0425 mg, 50µL) via nasal route using PE-10 tubing connected to micropipette (100 µL). LND suspension, SPANs and M-SPANs equivalent to 0.0425 mg LND was administered

via micropipette. After administration by intranasal route (0.0425 mg) at predetermined intervals (1h, 2h, 4h, 12h and 24h), the blood was collected retro orbitally (0.5 ml) from the animals in eppendorfs containing 30 µl of EDTA. Plasma was separated from the collected blood samples by centrifugation at 4000 rpm for 15 minutes. The plasma was carefully separated in an eppendorfs and stored at -20 °C until further analysis.

The rats were sacrificed humanely (intravenous administration of sodium pentobarbital (60mg/kg)) at different time intervals. Subsequently, the brain was dissected, washed twice using normal saline, made free from adhering tissue/fluid and weighed. The brain was weighed and an aliquot (1:10) of ice cold saline solution was added. The organs were then homogenized on ice. An aliquot of brain homogenates (100–500 µL) was extracted with acetonitrile (liquid–liquid extraction) and vortexed for 1 min [3]. After centrifugation (4 ^{0}C, 4000 rpm, 20 min), the supernatant was separated, and analyzed. The time intervals used for the study were 1h, 2h, 4h, 12h and 24h.

In order to clarify nose to brain direct transport more clearly, direct transport percentage (DTP) was calculated as follows;

$$\%DTP = \left(\frac{B_{in} - B_x}{B_{in}}\right) * 100$$

Where, $Bx = (B_{i.v}/P_{i.v}) \times P_{i.n}$.

- Bx is the brain AUC fraction contributed by systemic circulation through the BBB following intranasal administration.
- $B_{i.v}$ is the $AUC_{0–24\ h}$(brain) following intravenous administration.
- $P_{i.v}$is the $AUC_{0–24\ h}$ (blood) following intravenous administration.
- $B_{i.n}$ is the $AUC_{0–24\ h}$(brain) following intranasal administration.
- $P_{i.n}$ is the $AUC_{0–24\ h}$(blood) following intranasal administration.
- AUC is the area under the curve.

8.1.3.1.Preparation of the sample for analysis

For every sample, 250 µl plasma and 50 µl of a 0.2% solution of copper acetate were added into separate eppendorfs. The volumes in all eppendorfs were adjusted, as necessary, to 0.5 ml with

water. The eppendorfs were heated in a boiling water-bath for 15 min. and cooled. 0.5 ml of acetonitrile was added to each eppendorf and centrifuged for 15 min at 5000 rpm. Portions of 250 µl of the supernatant solutions were transferred into each of a set of eppendorfs followed by 150 µl of water. 100 µl of fluorescamine solution (0.05% w/v) was added to all eppendorfs. In the final reaction mixture, 0.5ml of each solution was taken in volumetric flasks and diluted up-to 5 ml with distilled water. The flask was kept at room temperature for 5 min. before analysis [3]. The resulting solutions were estimated by spectrofluorimetry at λ_{ex} =381nm and λ_{em}=494nm. The values of intensities were put into the equation obtained from calibration curve of drug in plasma (y = 45.675x + 0.6906) (details provided in analytical methods; section 3.7) and the final concentration of drug in plasma at different time points were calculated. Pharmacokinetic parameters were obtained using Kinetica software.

8.2.Nasal Penetration Study

Penetration of M-SPANs across nasal mucosa was studied using confocal microscopy. Fluorescein isothiocyanate (FITC) labelled M-SPANs suspension (equivalent to 0.0425 mg LND, 50µL), were administered into the rats nares (25 µL per nostril; n=6) using microlitre syringe attached with PE 10 tubing (100 µL). The tube was inserted at least 0.5 cm into the nostril to deposit the formulations inside the nasal cavity. The animals were sacrificed (intravenous administration of sodium pentobarbital (60mg/kg) after 15, 30, 60 and 120 min. After sacrificing the rats, nasal cavity of the rats was flushed with 5 ml 4% (v/v) formaldehyde in PBS pH 7.4. The region of epithelium most rich in olfactory neuron (dorsal and posterior in the nasal cavity) was excised and sheet of tissues were cut into pieces about 1mm square and immersed in the same fixative for at least 90 min [4]. A laser source for FITC at 470 nm was used to visualize the tissue (FSX100 (Olympus, USA)).

8.3. *In vivo* Nose to brain transfer of M-SPANs

The *in vivo* nose to brain transfer of M-SPANs (equivalent to 0.0425 mg LND, 50µL), was evaluated by quantitative estimation of iron in brain after intranasal administration in male Wistar rats (MSU/IAEC/2016-17/1602). M-SPANs were administered via intranasal route (n=6). Animals were sacrificed humanely (intravenous administration of sodium pentobarbital (60mg/kg) and brain was isolated after predetermined time points (15, 30, 60 and 120 min).

Brain was homogenized using a tissue homogenizer with 5ml of PBS and the homogenate was centrifuged at 15000 rpm for 30 min. The supernatant was collected and filtered through 0.45 µ filters and analyzed using ferrozine iron assay (Section 3.6). For qualitative estimation of nose to brain uptake of FITC labelled M-SPANs, animals were sacrificed humanely and brain was isolated after 60 min. The brain was covered with cryo-fixation liquid followed by cryosectioning to obtain 7µm thick brain slice. The brain slice was stained with DAPI for staining nucleus of brain cells. Brain slice was then observed under fluorescence microscope (FSX100 (Olympus, USA)).

8.4.Nasal toxicity study

Histopathological studies on isolated sheep nasal mucosa were conducted to assess the possible local toxic effects following nasal instillation of M-SPANs.

Sheep mucosa was obtained from slaughter house (Vadodara). Two separate nasal segments were carefully separated from the anterior and the posterior regions of the mucosa in the nasal cavity using forceps. Each segment was sectioned into three pieces. The pieces were treated with isopropyl alcohol as positive control (a), phosphate buffer saline (pH 6.4) as negative control (b), LND drug solution (0.0425 mg, 50µL) (c), and M-SPANs (equivalent to 0.0425 mg LND, 50µL) (d). After treatment for 2 hours, the pieces were washed with distilled water and preserved in 10% formalin solution for 48h. The histopathological studies were conducted according to the protocol described by Bancroft et al. [5]. The samples were dehydrated by treatment with serial dilutions of methyl alcohol, ethyl alcohol, and absolute ethyl alcohol, respectively. Specimens were cleared in xylene embedded in paraffin in a hot air oven. The temperature of the oven was adjusted at 56°C and the samples were kept for 24 h. Paraffin-beeswax tissue blocks were sectioned by a sledge microtome (Leica Microsystems SM2400, Cambridge, England). The obtained tissue sections (3–4 µm thickness) were collected, de-paraffinzed, stained by haematoxylin and eosin, and examined under a light microscope (Olympus, USA).

8.5.Biodistribution Study and Transfer of ^{177}M-SPANs to Brain After Nasal Administration

All animal studies were performed in compliance with guidelines set forth 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 and approved by the Animal Ethics Committee of ACTREC-TMC, Khargar, Navi Mumbai, India with registration no 106/GO/RcBi/S/99/CPCSEA

The pre-clinical biological evaluation of M-SPANs was done by carrying out biodistribution studies in normal Wistar rats using radiolabeled (Lutetium-177) M-SPANs.

8.5.1. Radiolabeling of M-SPANs

Labelling of ^{177}Lu was carried out in-house. For labelling, 1 mL of DOTA conjugated M-SPANs (2mg/mL) was diluted with 50µL of 1M acetate buffer at pH 5, followed by addition of 5µL of $^{177}LuCl_3$ solution. The mixture was incubated at 90 ^{0}C in a block heater for 45 min. After 45 mins, the nanoparticle dispersion was centrifuged at 16000 rpm for 15 mins. The obtained pellet was washed thrice with saline to removed unbound radioactivity. The pellet was dispersed in saline prior to administration to animals.

8.5.2. Biodistribution Study of ^{177}M-SPANs

^{177}M-SPANs were suspended in normal saline and administered via nasal route using PE-10 tubing (equivalent to 0.0425 mg LND, 50µL). Animals (n=6) were sacrificed (intravenous administration of sodium pentobarbital (60mg/kg) after 3h followed by excision of major organs and tissues (liver, intestine, stomach, kidney, heart, lungs, spleen, brain, muscle, blood, urine/stool), washed with saline, weighed and activity associated with them was measured in a flat type scintillation counter (Bhabha Atomic Research Centre). The distribution of the activity in different organs was calculated as per percentage of administered activity (dose) (%ID) per organ and percentage of administered activity per gram of the organ. The total uptake in blood, skeleton and muscle were calculated by considering that the respective tissue constitutes 7%, 10% and 40% of total body weight.

8.6. *In vivo* Mitochondrial Accumulation of M-SPANs

FITC conjugated M-SPANs and SPANs were suspended in normal saline and administered via nasal route using PE-10 tubing (equivalent to 0.0425 mg LND, 50µL). Animals (n=6) were sacrificed (intravenous administration of sodium pentobarbital (60mg/kg)) after 3h and brain was isolated. Brain homogenate was prepared by modifying previously described protocol to obtain

brain homogenate enriched in mitochondrial fraction [6]. The brain was washed three times with ice cold buffer (250mM sucrose, 5mM Tris, 1mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 0.1% fatty acid free bovine serum albumin (BSA), pH 7.4) and then flash frozen in liquid nitrogen. The brain was finely dissected into small fragments in a glass dish on ice. The pieces were diluted 1:10 in ice-cold brain homogenization medium (250 mM sucrose, 5mM Tris, 1mM EGTA, 0.1% fatty acid free bovine serum albumin (BSA), pH 7.4) and homogenized with overhead homogenizer. The homogenate was then centrifuged for 15 min at 600 g at 4 ^{0}C. Te supernatant was transferred into another tube and stored at -80 ^{0}C. Brain homogenate was exposed to rapid freeze thaw cycles before using it to measure activity of mitochondrial respiratory chain complex I, II, III and IV [7].

Complex I assay was assessed in assay mixture containing 25 mM potassium phosphate, 3.5g/l BSA, 2 mM ethylenediaminetetracetic acid (EDTA), 60 µM DCIP (2,6-dichloroindophenol), 70 µM decylubiquinone, 1 µM antimycin A and 0.2 nM reduced nicotinamide adenine dinucleotide NADH, pH 7.8. Rotenone sensitivity was calculated by subtracting activity of well using 10µM rotenone at 600 nm. For complex II activity, assay mixture containing 80 mM potassium phosphate, 1 g/l BSA, 2 mM EDTA, 80 µM DCIP, 10 mM succinate, 50 µM decylubiquinone, 1 µM antimycin A and 3 µM rotenone, pH 7.8. Absorbance was calculated at 600 nm. Maloanate sensitivity activity was calculated by subtracting the activity of wells with 20 mM malonate. Complex III was assessed using assay mixture containing 50 µM ferricytochrome C, 25 mM potassium phosphate, 4 mM sodium azide, 0.1 mM EDTA, 0.025% Tween 20 and 50 µM decylubiquinol, pH 7.4. Antimycin A sensitivity was calculated by subtracting activity of wells using 10 µM antimycin A by measuring absorbance at 550 nm. Complex IV activity was analyzed using assay mixture containing 30 mM potassium phosphate and 25 µM of freshly prepared ferricytochrome C, pH 7.4. The absorbance was calculated at 550 nm.

8.7. *In vivo* Toxicity of M-SPANs

All in vivo toxicity studies were planned according to standard guidelines and were approved by the Institutional Animal Ethics Committee, The Maharaja Sayajirao University of Baroda. The study design included six groups of six mice each caged separately and were treated intranasally, daily for fourteen consecutive days with 0.5 ml of test sample as follows:

Group 1 (negative control) – Milli Q; Group 2 (positive control) - 100 mg/kg body weight of ethylmethane sulfonate (through intra-peritoneal route), Group 3 (positive control)-100 mg/kg body weight of potassium dichromate (through nasal route), Groups 4–6 (Treatment group) Group 4: 10 mg/kg body weight of M-SPANs (through nasal route) Group 5: 25 mg/kg body weight of M-SPANs (through nasal route) Group 6: 50 mg/kg body weight of M-SPANs (through nasal route).

Animals were sacrificed humanely (intravenous administration of sodium pentobarbital (60mg/kg) after collecting blood (heart puncture) for biochemical assays. The liver tissues of all six groups were excised and washed in ice cold phosphate buffered saline (PBS) to remove superficial blood and stored at -80ºC for further study.

8.7.1. Blood Biochemical Analysis

Various serum biochemistry parameters, including liver function markers such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) were determined to assess liver dysfunction caused by M-SPANs [8].

8.7.2. Oxidative Stress Markers

A 10% tissue homogenate was made in 0.1 M phosphate buffer (pH 7.4) containing 0.1 M KCl. The enzyme assays were performed with the supernatant obtained after centrifugation of the homogenate at 9000 x g for 10 min at 4ºC. Protein was measured by the method of Bradford (1976) using bovine serum albumin standard [9]. Glutathione was estimated by using previously reported method [10]. Briefly, 1 ml of 5% Trichloroacetic acid was added to an equal volume of cell lysate for in vitro or 10% homogenate for in vivo studies, and incubated at room temperature for 30 min. It was then centrifuged at 250 x g for 15min. 2.5 ml of 5-5'-dithiobis (2-nitrobenzoic acid) reagent was added to 0.5 ml of supernatant, and kept for incubation for 15min. It was then read at 412 nm. Different concentrations of glutathione were taken as standard and data represented as µmoles/gm tissue (for in vivo) and µmoles/mg protein (for in vitro) [10]. For estimation of lipid peroxidation, tissue homogenate (10%) was prepared in 0.1 M phosphate buffer in tissue homogeniger. To 1 ml of homogenate, 2 ml of TCA (trichloroacetic acid)-TBA (thiobarbituric acid)-HCl reagent (10% TCA and 0.67% TBA dissolved in 0.25 mol/L of HCl) was added and mixed thoroughly. The solution was heated in a boiling water bath for 30 min.

After cooling at room temperature, the precipitate was removed by centrifugation (1000 x g, 10 min) [11]. The absorbance of the supernatant was read at 530 nm. The malondialdehyde concentration in the sample was calculated using an extinction coefficient of 1.56 x 105 L/mol and was expressed as nano moles malondialdehyde/g tissue using standard curve of 1, 1, 3, 3-tetramethoxypropane.

8.8.Interaction with Mucin, Plasma and Extracellular Matrix Protein

Interaction of M-SPANs with mucin, plasma and extracellular matrix protein was done according to method described in section 5.16.

8.9.Results and Discussion

8.9.1. Pharmacokinetic Study

The results of pharmacokinetic study are shown in Table 8.2. From the result we can observe that SPANs and M-SPANs greatly altered the pharmacokinetic profile of LND when compared to plain drug suspension (Figure 8.1).

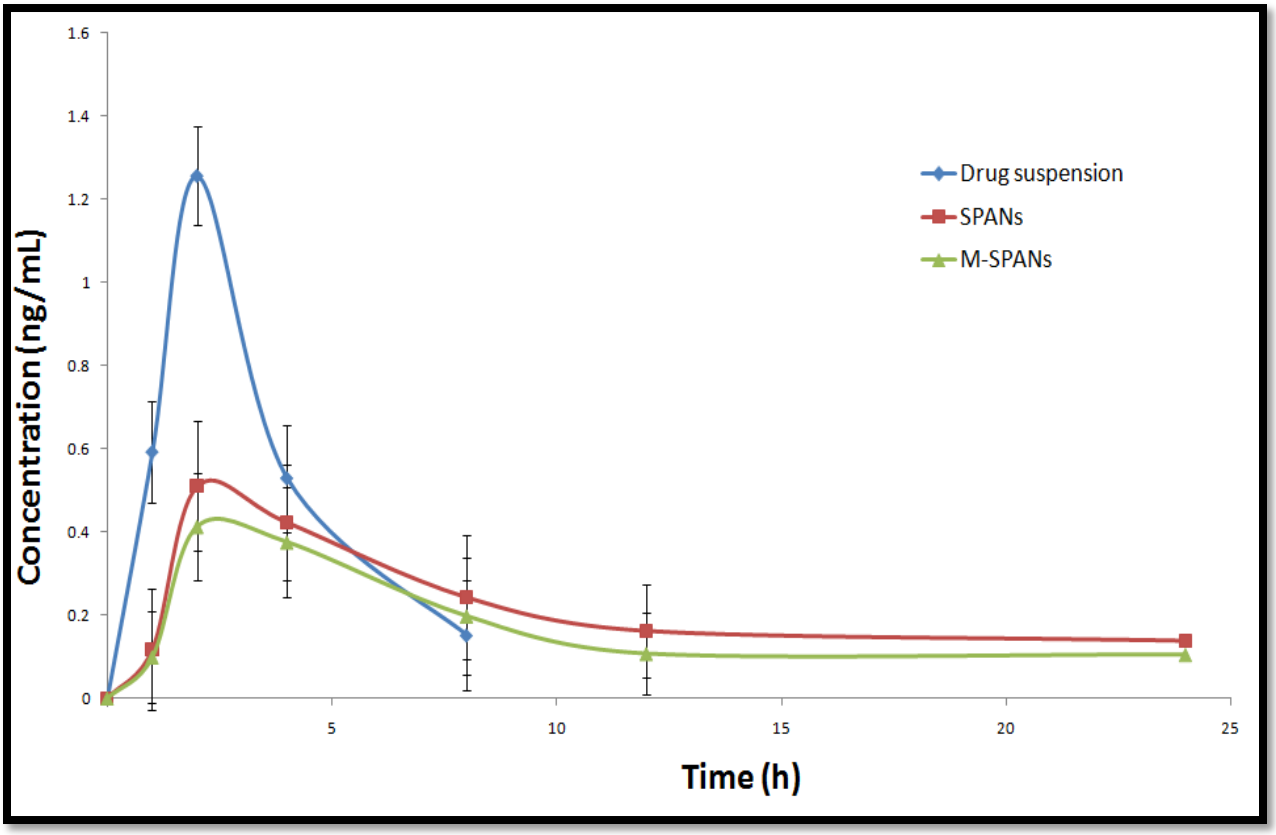

Figure 8.1: Concentration of drug in plasma after administration by intranasal route

Table 8.2: Concentration of LND in rat plasma after intranasal administration of drug suspension, SPANs and M-SPANs

Time (hr.)	Drug suspension (i.n.) (ng/ml) ± SD	SPANs (i.n.) (ng/ml) ± SD	M-SPANs (i.n.) (ng/ml) ± SD
0	0	0	0
1	0.592±0.121	0.118±0.147	0.991±0.111
2	1.255±0.119	0.510±0.156	0.413±0.128
4	0.529±0.132	0.423±0.151	0.375±0.133
8	0.065±0.129	0.242±0.139	0.197±0.141
12	-	0.162±0.148	0.138±0.125
24	-	0.107±0.112	0.105±0.098

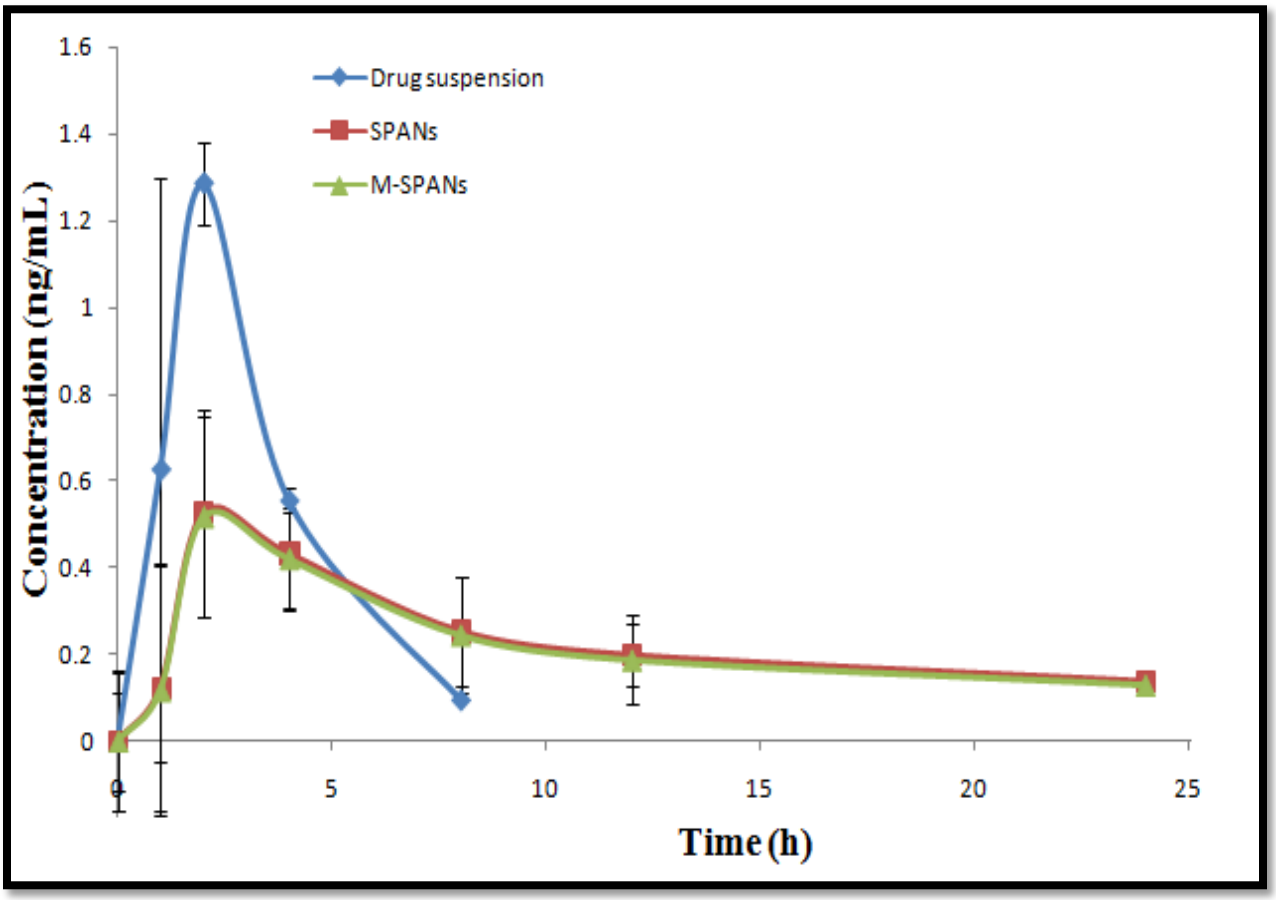

Figure 8.2: Concentration of drug in plasma after administration by intravenous route

Table 8.3: Concentration of LND in rat plasma after intravenous administration of drug suspension, SPANs and M-SPANs

Time (hr.)	Drug suspension (i.v.) (ng/ml) ± SD	SPANs (i.v.) (ng/ml) ± SD	M-SPANs (i.v.) (ng/ml) ± SD
0	0	0	0
1	0.626 ±0.112	0.123 ±0.159	0.116 ±0.161
2	1.285 ±0.672	0.525 ± 0.284	0.517 ± 0.288
4	0.554 ±0.096	0.432 ± 0.241	0.421± 0.231
8	0.095 ±0.029	0.253 ± 0.126	0.244 ± 0.118
12	-	0.198 ± 0.128	0.187 ± 0.132
24	-	0.137 ± 0.071	0.129 ± 0.102

Table 8.4: Comparative pharmacokinetic parameters of LND delivered in suspension, SPANs and M-SPANs via intranasal route

Parameters	Drug suspension (IN)	SPANs (IN)	M-SPANs (IN)
T_{max}(h)	1.975±0.119	3. 922±0.124	3.917±0.133
AUC (ng/ml*h)	2.748±0.139	6.118±0.217	6.195±0.224
MRT (h)	3.789±0.190	9.627±0.231	9.586±0.238
C_{max}	1.263±0.631	0.510 ± 0.281	0.413 ± 0.257
T_{half}(h)	1.905±0.441	6.743±0.437	6.775±0.452

Table 8.5: Comparative pharmacokinetic parameters of LND delivered in suspension, SPANs and M-SPANs via intravenous route

Parameters	Drug suspension (IN)	SPANs (IN)	M-SPANs (IN)
T_{max}(h)	1.557±0.144	8.673±0.129	8.744±0.121
AUC (ng/ml*h)	3.777±0.149	5.753±0.137	5.671±0.159
MRT (h)	3.527±0.153	8.682±0.118	8.527±0.133

From the Table 8.2 and 8.3, we can observe similar pharmacokinetics of SPANs and M-SPANs when administered via intravenous route. This can be attributed to the fact that in case of both SPANs and M-SPANs, the conjugation of drug is similar and hence their release profile shows

minimum variation. From Table8.4, it can be seen that there was significant ($p<0.05$) increase in half-life of drug in SPANs and M-SPANs (6.7431±0.437 and 6.7757±0.452 respectively) in comparison to drug suspension (1.9054±0.441). The area under curve (AUC) was also significantly higher ($p<0.05$) for SPANs and M-SPANs (5.2186±0.217 and 5.1957±0.224 respectively) in comparison to drug suspension (2.7487±0.139). The Mean Residence Time (MRT) of drug was also found to be significantly higher ($p<0.05$) for SPANs and M-SPANs (9.627±0.231 and 9.5861±0.238 respectively) compared to drug suspension (3.7898±0.190). Higher mean residence time and half-life of drug in SPANs and M-SPANs may be due to the conjugation of drug with hyaluronic acid [4]. Pharmacokinetic studies indicated that the AUC was higher for SPANs and M-SPANs as compared to plain drug suspension. This may be attributed to drug-HA conjugates present in both SPANs and M-SPANs. Larger AUC will further help for better tumor accumulation and correspondingly improved antitumor efficacy as compared to free drug [5]. SPANs and M-SPANs enhanced the maximum drug concentration (*C*max) and the area under the concentration–time curve (AUC) compared with LND solution administered by intranasal route ($p< 0.01$). As prolonged plasma circulation is the driving force for increased tumor targeting, nanoparticulate drug delivery system through intranasal route is expected to demonstrate improved therapeutic efficacy. No significant change in pharmacokinetic parameter was observed between SPANs and M-SPANs. This may be due to the fact that in both SPANs and M-SPANs, the drug was conjugated in similar manner and hence both nanoparticles demonstrate similar drug release behavior. M-SPANs were selected for further *in vivo* studies as it demonstrated activity similar to SPANs but possess additional ability for efficient mitochondrial localization to enhanced tumor suppression efficacy.

8.9.2. Brain Targeting Efficiency

The pharmacokinetic parameters of M-SPANs after intranasal and intravenous administration can be observed from Table 8.4 and 8.5. When M-SPANs after intranasal and intravenous administration were compared, higher AUC was observed with intranasal administration in brain.

The drug targeting percentage (DTP %) represents the percentage of drug directly transported to the brain via the olfactory pathway (Table 8.6). The M-SPANs demonstrated %DTE of 64.11±4.68% and % DTP of 74.34 ±3.76% suggesting efficient brain targeting ability of M-

SPANs. Thus, the results of the present investigation prove that LND could be transported directly to the CNS after intranasal delivery of M-SPANs, thereby enhancing drug concentration in the brain.

Table 8.6: Pharmacokinetics parameters of M-SPANs after intranasal and intravenous administration

Pharmacokinetic parameters	**Brain**		**Blood**	
	Intranasal	Intravenous	Intranasal	Intravenous
$t_{max(h)}$	2	3	3.5	2
C_{max} (ng/ml)	0.734±0.227	0.412±0.143	0.534±0.092	0.813±0.186
AUC $_{o\rightarrow 8}$(ng.h/ml)	5.65±0.665	2.14±0.132	3.34±0.724	6.75±0.965
$t_{1/2}$(h)	3.74	3.19	4.92	6.62
MRT(h)	6.28	6.10	7.42	10.35

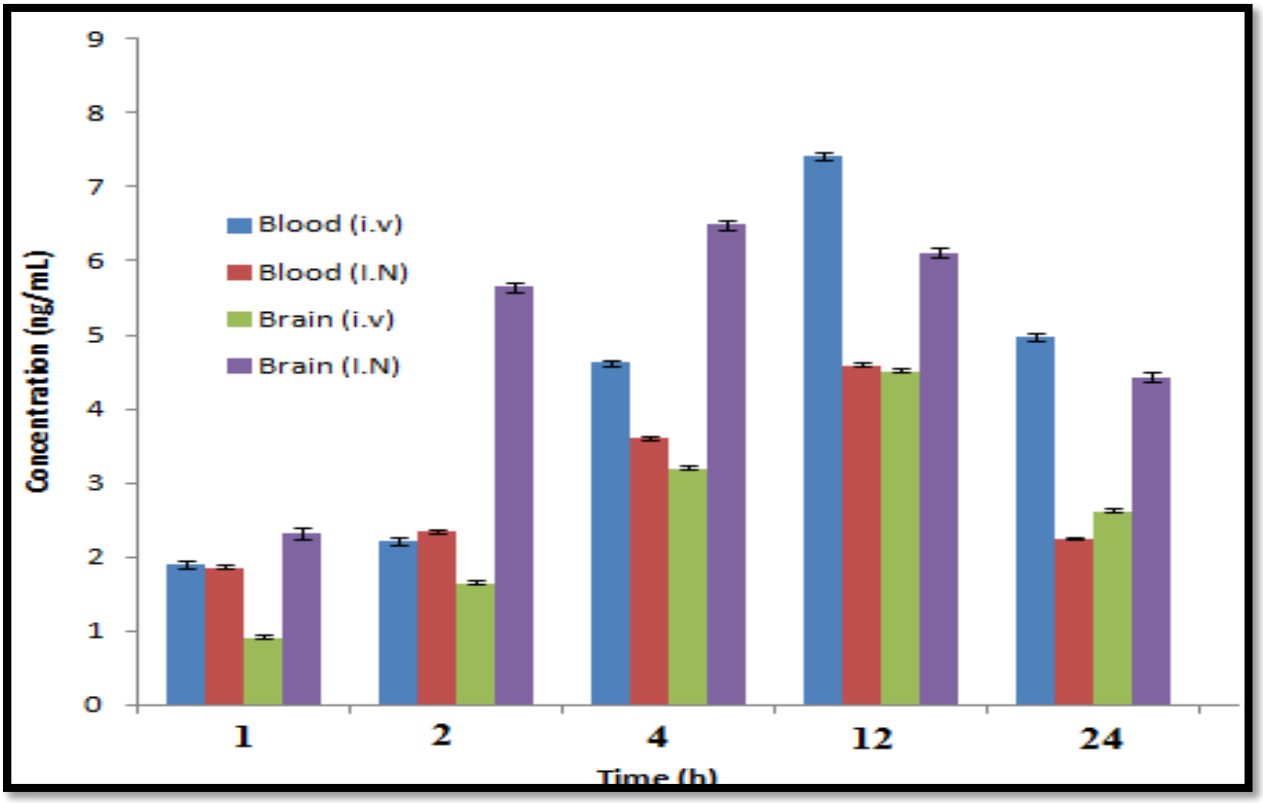

Figure 8.3: A comparative bar graph representation of drug concentration v/s time profile in blood and brain after intranasal and intravenous administration of M-SPANs

8.9.3. Nasal Penetration Study and *In vivo* Nose to brain transfer of M-SPANs

Figure 8.4(1) shows the penetration of M-SPANs across the nasal epithelium after 15, 30, 60 and 120 minrespectively in form of fluorescence intensity. Figure 8.4 (2A, 2B, 2C and 2D) shows the penetration of M-SPANs across nasal epithelia (olfactory epithelia) in 15, 30, 60 and 120 min respectively.

The results demonstrate that M-SPANs have efficient olfactory uptake. The high fluorescence reveals significant accumulation of M-SPANs which can significantly increase accumulation of drug in brain and tumor because Ctx has been also demonstrated to be overexpressed in glioma cells.

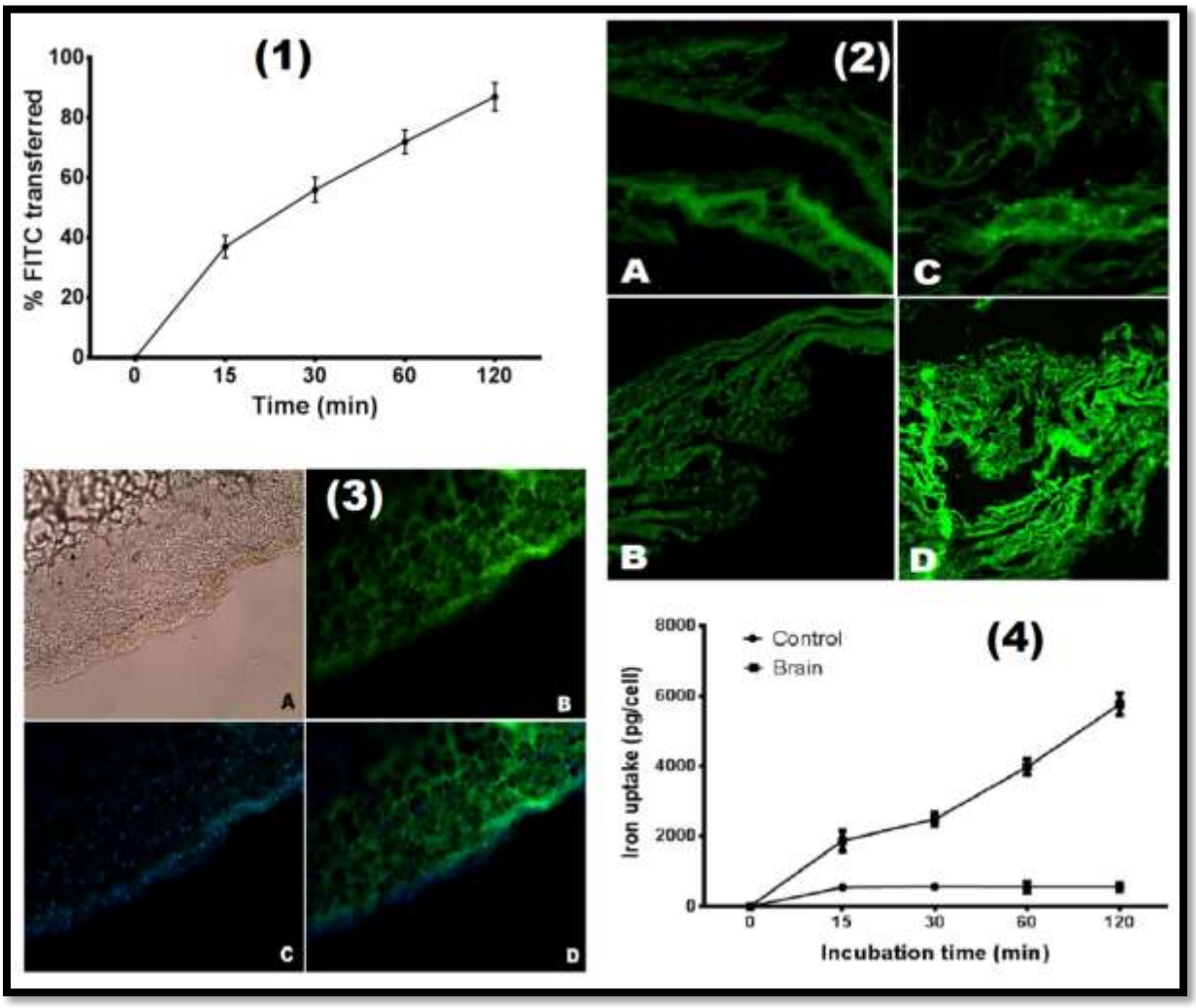

Figure 8.4: Percent transfer of FITC conjugated M-SPANs across nasal mucosa (1), *in vivo* nasal penetration of M-SPANs (2A-D) after 15, 30, 60 and 120 min respectively. Accumulation of M-SPANs in brain after nasal administration was assessed qualitatively after 15 min (8.4-3(A)), 30 min (8.4-3(B)), 60 min (8.4-3(C)) and 120 min (8.4-3(D)) using fluorescence microscopy, while quantitative estimation of SPANs in brain was done using ICP-AES (8.4-4).

The M-SPANs brain targeting ability was evaluated by a real time brain distribution analysis under an in vivo imaging system at 0.25 to 2 h after intranasal administration. Figure 8.4 (3) shows qualitative uptake of M-SPANs in brain after 60 min. Figure 8.4 (3A, 3B,3C and3 D) represents the bright field image of the brain cross-section, fluorescence from FITC conjugated M-SPANs accumulated in brain, DAPI stained cryosection of brain and overlay of 3B and 3C respectively.

The results demonstrate significant accumulation of M-SPANs in brain after 60 min which confirm efficient nose to brain transfer of M-SPANs via olfactory neurons. The quantitative estimation of M-SPANs after various time intervals (15, 30, 60 and 120 min) is shown in figure 8.4-4. The amount of M-SPANs in brain increased after 30min as it is the time lag required for nose to brain transfer of nanoparticles [6]. The results suggest M-SPANs as an efficient system for nose to brain delivery of drug.

Enhanced accumulation of M-SPANs after intranasal administration might be due to direct transport of SPANs to the brain via both the olfactory and the trigeminal nerves pathway. In addition to direct nose-to-brain transport, Ctx which is conjugated to M-SPANs might act as substrate for MMP-9 receptor which is highly expressed in brain tumor endothelial cells [7] and has been proved efficient in facilitating the selective uptake by glioma cells.

8.9.4. Nasal Toxicity

The histopathological condition of nasal mucosa after treatment with isopropyl alcohol as positive control (a), PBS (pH 6.4) as negative control (b), LND drug solution (c) and M-SPANs (d) was observed to confirm the safety of M-SPANs for nasal administration (Figure 8.5). Neither cell necrosis nor removal of the cilia from the nasal mucosa was observed after treating with M-SPANs. These observations indicate that the M-SPANs did not cause any deleterious response and adverse effect on nasal mucosa. The epithelial layer was intact, and there were no

alteration in the basal membrane and the superficial part of the sub mucosa compared with PBS-treated mucosa (negative control) (Figure 8.5b). In the case of nasal mucosa treated with drug solution (Figure 8.5c), some cilia got detached; this indicates drug toxicity on the nasal mucosa. Mucosa treated with isopropyl alcohol (positive control) (Figure 8.5a) showed heavy loss of epithelial cells and shrinkage of the mucosal layer of epithelial tissues. Thus, these results indicate that the M-SPANs (Figure 8.5d) seem to be safe for nasal administration.

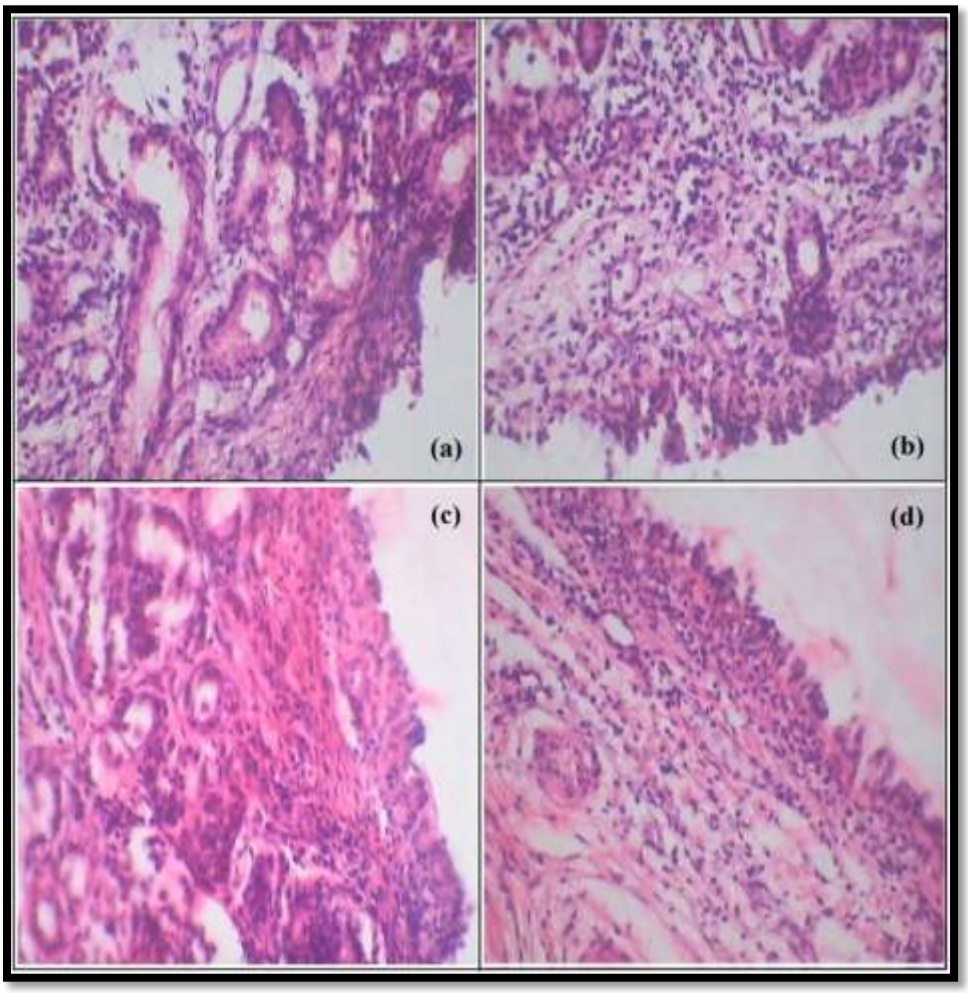

Figure 8.5: Histopathological images of isopropyl alcohol as positive control (a), PBS (pH 6.4) as negative control (b), LND drug solution (c) and M-SPANs (d)

8.9.5. Biodistribution Study and Transfer of ^{177}M-SPANs to Brain After Nasal Administration

The results of biodistribution studies confirm the transfer of M-SPANs from nasal cavity into brain within 3h of administration (Table 8.7). The % administered activity found in brain was approximately 22%. As suggested by the result, we can observe that around 50% of activity was observed in stomach which was due to transfer of M-SPANs from nasal cavity into food pipe. If we subtract amount of activity found in stomach owing to limitation of device for nasal administration, biodistribution result demonstrated that almost 50% of administered activity from nasal cavity was transferred into brain. The result suggested that M-SPANs can efficiently deliver drug to brain via nasal cavity. This ^{177}Lu based M-SPANs can also be a potential brain targeted theranostic agent as the ^{177}Lu possess both therapeutic as well as imaging ability which can be utilized for gamma scintigraphy counting as well as radio-luminescence imaging.

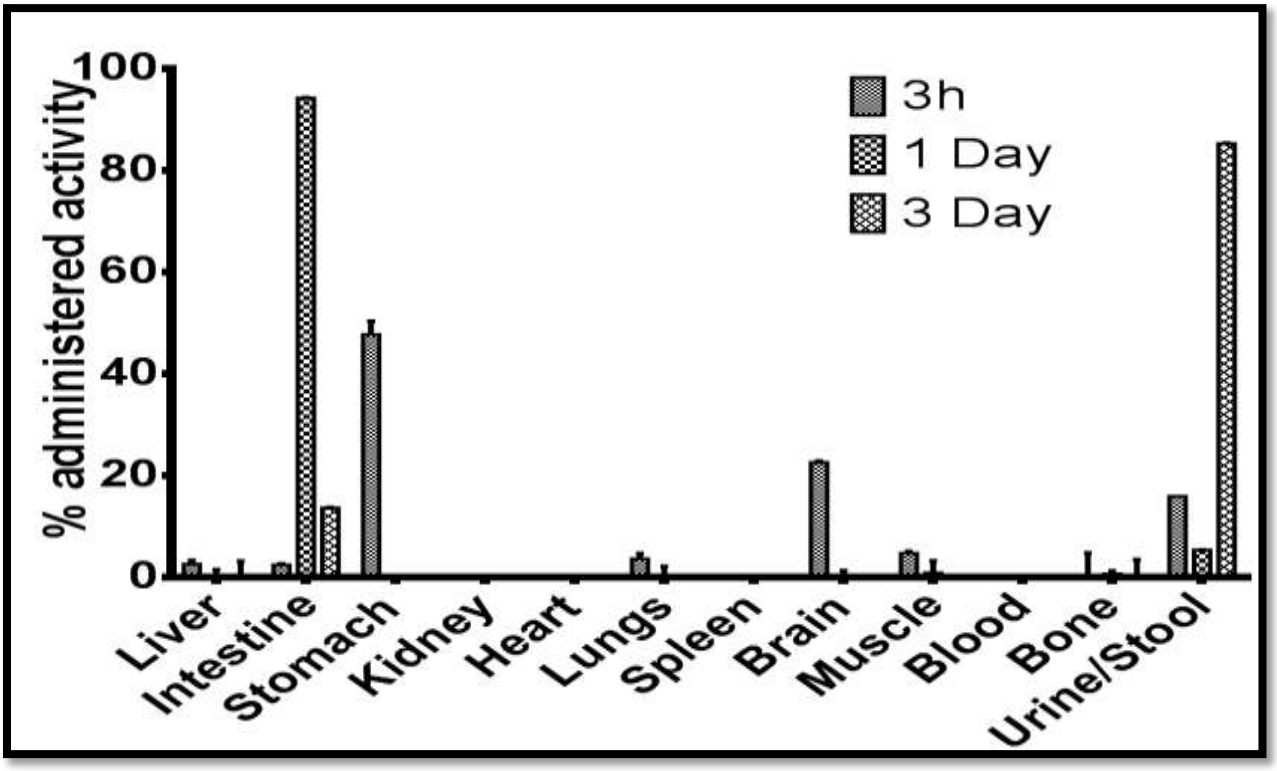

Figure 8.6:% administered activity after 3h, 1 day and 3 day in rats

Table 8.7**:** Biodistribution pattern of ^{177}M-SPANs administered via intranasal route

Organ	**3hr (%ID/g)**	**1D (%ID/g)**	**3D (%ID/g)**
Liver	2.59±0.73	0.02±0.44	0.04±0.01
Intestine	2.40±0.27	94.29±0.04	16.56±0.1

Stomach	47.76±2.6	0.19±0.03	0.14±0.03
Kidney	0.07±0.14	0.03±0.0	0.15±0.04
Heart	0.00±0.01	0.02±0.07	0.04±0.01
Lungs	3.63±1.05	0.02±0.09	0.06±0.0
Spleen	0.15±0.0	0.01±0.01	0.06±0.01
Brain	22.63±0.25	0.04±0.02	0.11±0.01
Muscle	4.71±0.38	0.89±0.31	0.00±0.02
Blood	0.00±0.29	0.11±0.27	0.24±0.16
Bone	0.00±0.47	0.64±0.59	0.45±0.03
Urine/Stool	15.94±0.0	5.35±0.01	82.55±0.05

From the result of biodistribution study performed using ^{177}M-SPANs, we can observe that the presence of ^{177}M-SPANs was dominant in brain in the first 3h (22.63%±0.25%) after administration followed by stomach (47.76%±2.6%) which is due to drainage of ^{177}M-SPANs into stomach via nasal drainage passage. After 1 day (1D) of ^{177}M-SPANs administration, 94.29±0.04% of injected activity was observed in intestine while injected activity decreased drastically in both brain and stomach. Maximum injected activity after 3 days (3D) was observed in urine/stool (82.55±0.05%) followed by intestine (16.56±0.1%). The obtained result demonstrated clearance of M-SPANs after 3 days. An important finding was the minimum accumulation of M-SPANs in liver. Maximum injected activity was observed after 1h (2.59±0.73%). After 1 day no significant injected activity was observed in liver confirming minimum chances of liver toxicity caused due to accumulation of nanoparticles in liver [8].

8.9.6. *In vivo* Mitochondrial Accumulation of M-SPANs

Mitochondrial accumulation of M-SPANs in brain cells was determined with respect to effect of SPANs and M-SPANs on enzymatic activity of individual mitochondrial respiratory chain complexes in brain homogenate. Cryosectioning of brain was done and visualized under fluorescence microscope prior to homogenization to confirm brain accumulation of M-SPANs and SPANs (Figure 8.7).

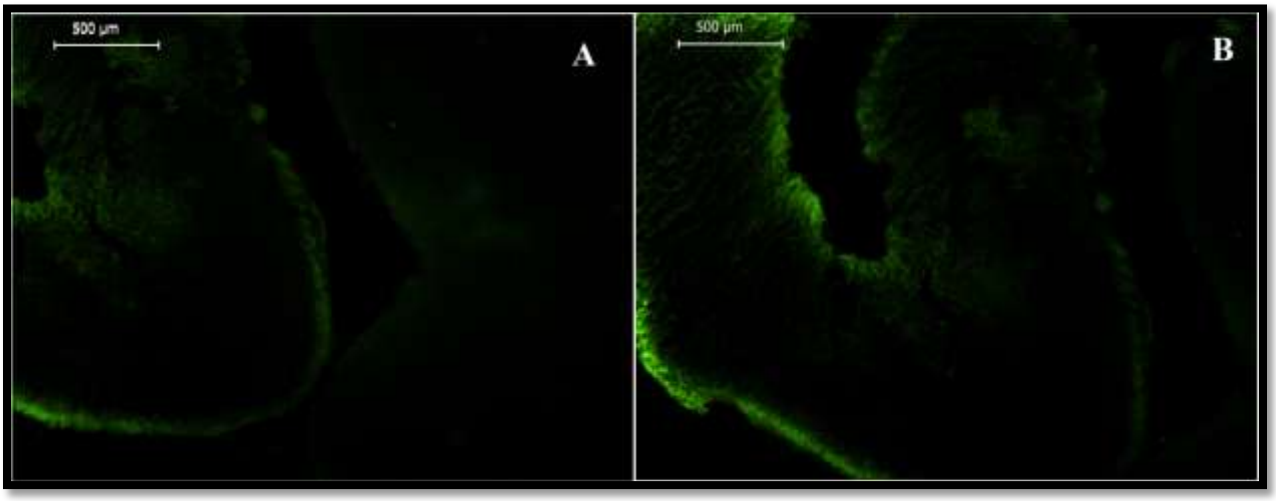

Figure 8.7: The fluorescence image confirmed accumulation of SPANs (Figure 8.7A) and M-SPANs (Figure 8.7B) in brain after 3h.

The enhanced accumulation of M-SPANs can be observed in comparison to SPANs. This may be due to presence of TPP ion on surface of M-SPANs which enhances penetration across lipid bilayers. The effect of SPANs and M-SPANs on mitochondrial chain complexes demonstrates enhanced suppression of mitochondrial respiratory chain complexes by M-SPANs in comparison to SPANs (Figure 8.8).

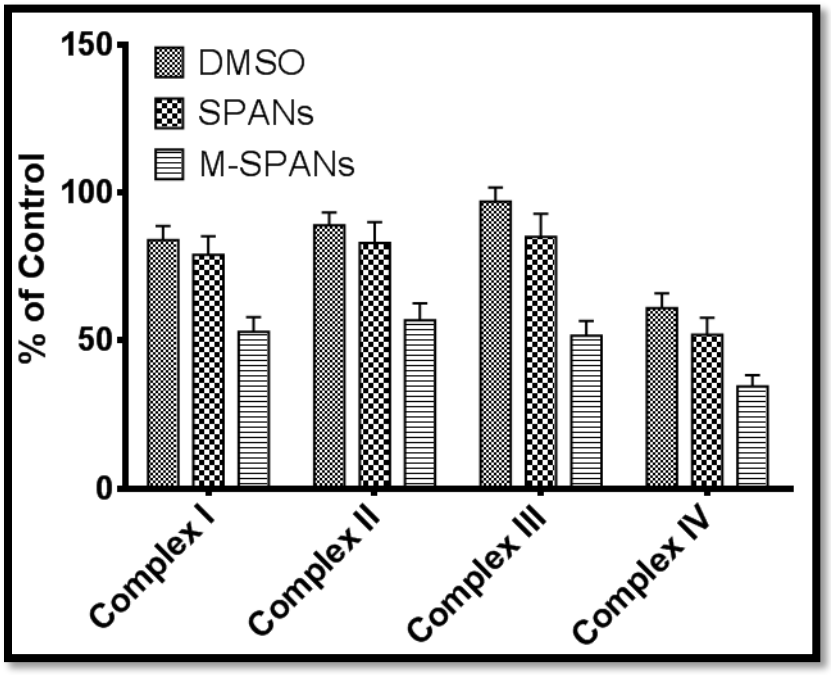

Figure 8.8**:** Activity of mitochondrial respiratory chain complexes in brain homogenate in presence of SPANs and M-SPANs.

Fig. 8.8 shows a significant inhibitory effect of M-SPANs on all four respiratory complexes i.e. complex I (53.1±2.7), complex II (56.8±4.3), complex III (49.7±4.8) and complex IV (34.5±3.9). Complex III and IV appeared to be the most sensitive respiratory chain complex. SPANs inhibited the respiratory complex less significantly (79.2±4.3, 83.1±4.7, 84.9±4.9, 52.7±3.8) than M-SPANs. A plausible explanation of these observations may be correlated to incorporation of TPP molecule in the inner mitochondrial membrane. Since the respiratory complexes are known to be sensitive to their lipid environment and require phospholipid molecule for their activity, a high proportion of TPP molecule in the membrane could impair insulating property of membrane allowing protons to leak back into the matrix and the membrane structure required for functioning of the protein complexes.

8.9.7. *In vivo* Toxicity of M-SPANs

In this work, neither mouse death nor obvious abnormality was noticed for mice after intranasal administration of M-SPANs at a highest dose of 50 mg/kg within 14 days. The blood analysis result demonstrated significant increase in levels of AST at the highest dose of 50 mg/kg body weight (Table 8.8). Although increase in the levels of ALP and ALT was observed at all the three doses of M-SPANs treated groups (10, 25 and 50 mg/kg body weight) as compared to control group, no significant increase was observed at dose of 10 mg/kg body weight.

Table 8.8: Effect of M-SPANs on liver function

Sr. No	Condition	Control (Milli Q)	M-SPANs (mg/kg body weight)		
			10	25	50
1	ALT (U/L)	38.26±1.2	41.59±1.9	43.96±2.4	54.25±2.7
2	AST (U/L)	128.55±1.7	168.54±2.7	173.22±3.9	213.45±4.1
3	ALP (U/L)	187.65±2.1	193.70±3.3	194.12±3.5	201.67±3.7

The level of glutathione (GSH) level in liver was found to significantly decrease at highest dose exposure of 50 mg/kg body weight after 14 days as compared to the control mice (Table 8.9).

Similar to glutathione, MDA levels in hepatic cells were also significantly increased at highest dose of 50 mg/kg body weight.

Table 8.9: Effects of M-SPANs on glutathione and lipid peroxidation levels in mouse liver

Sr. No	Groups	GSH (µM/g Tissue wt)	MDA (n mol/g Tissue wt)
1	Control	3.85±0.55	65.51±1.3
2	M-SPANs (10 mg/kg body weight)	3.69±0.17	71.40±3.9
3	M-SPANs (25 mg/kg body weight)	3.42±0.25	83.20±1.6
4	M-SPANs (50 mg/kg body weight)	3.17±0.31	94.45±2.2

The results of liver functions and oxidative stress marker suggested that at highest dose of M-SPANs (50mg/kg body weight) does lead to significant changes in liver function of mouse. Although no mortality was observed after 14 days, the maximum dose of M-SPANs tolerated without significant changes in liver functions should be less than 50mg/kg body weight.

8.10. Interaction with Mucin, Plasma and Extracellular Matrix Protein

The interaction of nanoparticles in various biological media plays an important role in deciding the fate of nanoparticles and their delivery purpose. The interaction of SPANs with mucin was done to assess the change in surface conditions of SPANs in presence of mucin, ECM and plasma (Table 8.10) as shown in Figure 8.9.

Table 8.10: Assessment of interaction between M-SPANs and bio-molecules (plasma, mucin and ECM)

Sr. No.	Nanoparticles	Particle size	Zeta potential
1	M-SPANs	17.4±2.3	31.7±3.7
2	M-SPANs + Mucin	21.7±2.8	34.2±3.2
3	M-SPANs + ECM	23.9±2.6	28.7±3.1
4	M-SPANs + Plasma	20.1±1.9	24.5±3.7

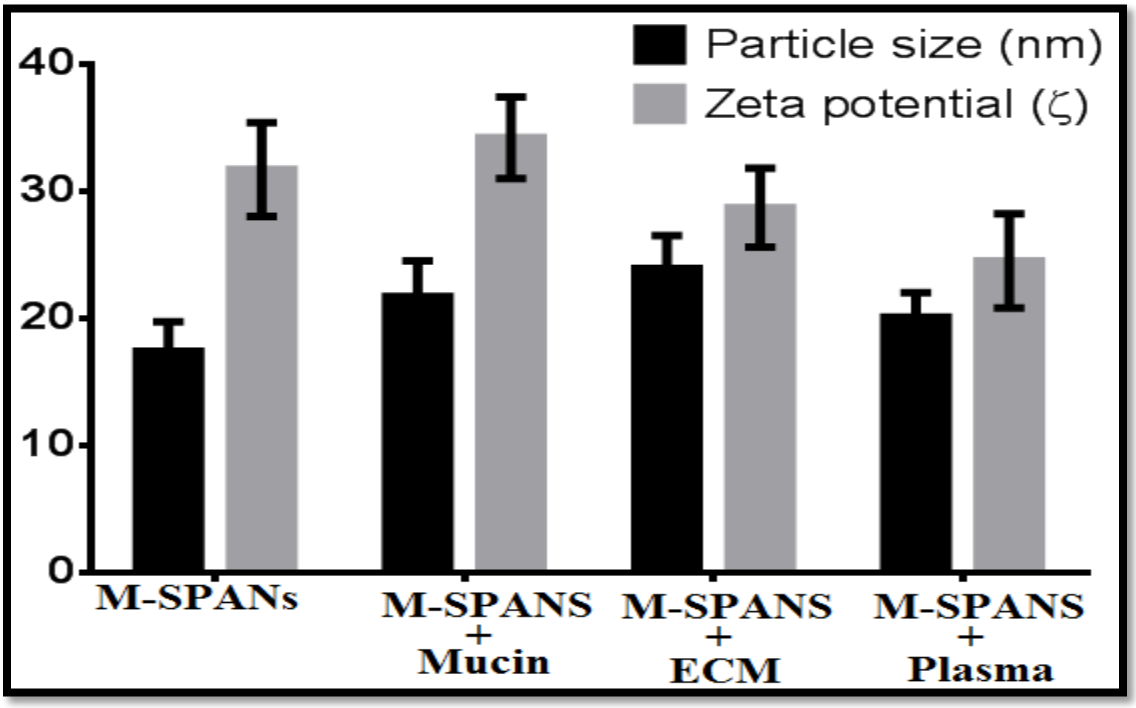

Figure 8.9: Assessment of interaction between M-SPANs and bio-molecules (plasma, mucin and ECM)

The result suggested absence of significant interaction between mucin (derived from bovine) and M-SPANs as no significant change in zeta potential or particle size was observed. The variation observed in zeta potential and particle size can be attributed to the interaction between negatively charged sialic acid residue of mucin and positively charged M-SPANs [9]. Similar results were observed in case of interaction with ECM and plasma. The insignificant interaction between M-SPANs and mucin demonstrated that the M-SPANs won't get entangled in mucin which can hamper the mucus penetration of M-SPANs. The interaction with ECM was studied to assess the effect of ECM protein on M-SPANs. If the M-SPANs undergo significant interaction with ECM protein in CSF, M-SPANs will lose their surface functionality due to formation of protein corona which will ultimately lead to decreased accumulation of M-SPANs in tumor. The results demonstrate absence of any significant interaction with biological media and hence M-SPANs can act as an effective platform for targeting tumor without losing their surface functionality.

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