

## Chapter 8                      Animal studies: Pharmacokinetic, biodistribution and toxicity studies of developed TNPs, HA-TNPs and CS-TNPs

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### 8.0    *In vivo* studies

All experimental protocol for *in vivo* studies were reviewed and approved by Institutional Animal Ethics Committee, Pharmacy department, The M. S. University of Baroda, Vadodara, vide protocol approval no: MSU/IAEC/2018-19/1830. All the experimental procedures were carried out as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines released by Ministry of Environment, Forests and Climate Change and Ministry of Social Justice and Empowerment, Government of India, New Delhi, India. All the *in vivo* experiments were performed on Wistar rats. Wistar rats (any sex) of 150-200 g were obtained from official CPCSEA breeder. The rats were housed in cages placed in an animal room with a constant temperature of 22 °C and a fixed 12-hour light-dark cycle. All animals were handled and housed according to the guidelines and manual set by the Committee of the Care and Use of Laboratory Animals. The rats were given standard rat chow ad libitum and water. After acclimatization the studies were performed as follows:

### 8.1    Methods

#### 8.1.1    Pharmacokinetic study

Pharmacokinetic study on Wistar rats were performed to obtain plasma concentration-time profile of TMZ, TNPs, HA-TNPs and CS-TNPs. Thirty Wistar rats (150–200 g) were randomly divided into five groups (n=6) and were fasted over night before the experiment but allowed to drink water freely. Animals in group I were control, animal in group II received pure TMZ dispersion, the animals in group III received TNPs, animals in group IV received HA-TNPs and animals in group V received CS-TNPs by intravenous route via tail vein at a dose of 3 mg/kg of TMZ (1). At predetermined intervals (0.5h, 1h, 3h, 6h, 12h, 24h, 48h, 60h and 72h), blood samples were collected retro orbitally in EDTA containing tubes and centrifuged at 5000 rpm for 20 min by a cooling centrifuge (Remi Equipments Ltd). The separated plasma was stored at -20 °C until further analysis (2).

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### Parameters:

- Plasma concentration versus time was plotted and pharmacokinetic parameters were determined after dose normalization.
- $C_{\max}$  was directly determined from the concentration–time profile.
- Mean residence time (MRT) and elimination rate constant ( $K_{el}$ ) for the drug were determined from the plot.
- Area under the curve (AUC) was calculated by the trapezoidal rule

### 8.1.2            Biodistribution study

Wistar rats were randomly divided into five groups with six animals for each time point. Rats in group I received saline solution (control group), group II received pure TMZ dispersion, animals in group III, IV and V received TNPs, HA-TNPs and CS-TNPs at a dose of 3 mg/kg of TMZ via tail vein. At predetermined time intervals (12h and 24h), rats were sacrificed by euthanization and different organs like brain, heart, lung, liver, kidney and spleen were isolated and blood was collected. Blood samples were processed as mentioned earlier in pharmacokinetic study. Organs were rinsed and homogenized with cold saline by tissue homogenizer, centrifuged (5000 rpm × 20 min) at 4°C and stored at -20 °C in till further analysis. The TMZ level in plasma and different organs was determined by HPLC analysis (3).

### 8.1.3            In vivo toxicity study

Rats were randomly divided into five groups (n=6 in each group). Saline solution, pure TMZ, TNPs, HA-TNPs and CS-TNPs at a dose equivalent to 30 mg/kg were administered intravenously via tail vein to different group animals. After dose administration, all the animals were maintained on water and normal diet. After 7 days, blood was collected retro-orbitally and subjected to biochemical parameter estimation viz. blood parameters and clinical parameters using standard kits through standard procedures in an automated bioanalyser (3,4).

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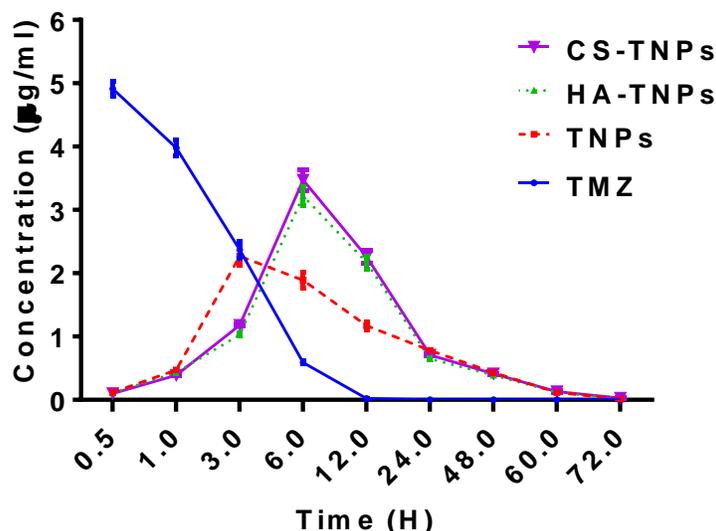
### 8.2 Result and discussion

#### 8.2.1 In vivo pharmacokinetic study

The results of pharmacokinetic study (figure 8.1) indicated significant increase in mean plasma drug levels after i.v. administration of TNPs, HA-TNPs and CS-TNPs as compared to pure TMZ (Table 8.1). TMZ showed  $C_{max}$  of  $4.9 \pm 0.1 \mu\text{g/ml}$  at 0.5 h and then level of TMZ was found to be maintained up to 12 h. The  $C_{max}$  of  $2.27 \pm 0.12$ ,  $3.2 \pm 0.1 \mu\text{g/ml}$  and  $3.47 \pm 0.11 \mu\text{g/ml}$  was observed for the TNPs, HA-TNPs and CS-TNPs at  $T_{max}$  of 3.0 h and 6.0 h respectively. As compared to TNPs, HA-TNPs and CS-TNPs, TMZ was quickly removed from the circulation. No TMZ was detected after 12 hrs. TMZ was still detected in the plasma until 72 hr post injection of TNPs, HA-TNPs and CS-TNPs. The difference in obtained results of TMZ and nanoparticles (TNPs, HA-TNPs and CS-TNPs) may be due to slow diffusion of TMZ from nanoparticles which continued for more than 72 h (2). Student t-test showed significant differences in the mean concentration of TMZ in plasma after i.v. administration of pure TMZ, TNPs, HA-TNPs and CS-TNPs at all time points. TNPs, HA-TNPs and CS-TNPs showed significant enhancement in the pharmacokinetic profile of TMZ as compared to plain drug solution, in terms of Area Under Curve (AUC), half life ( $t_{1/2}$ ) and mean residence time (MRT). The AUC of TNPs, HA-TNPs and CS-TNPs was approximately 2.8 folds, 3.3 folds and 3.5 folds higher than pure TMZ respectively while the  $t_{1/2}$  of TMZ was approximately 3.4 folds, 3.3 fold and 4.9 folds increased when administered as TNPs, HA-TNPs and CS-TNPs. Enhancement in  $t_{1/2}$  may be due to the small particle size of TNPs, HA-TNPs and CS-TNPs. The small particle size significantly reduces the affinity of macrophages thus prolonging the  $t_{1/2}$  of TMZ *in vivo* (5). The  $t_{1/2}$  prolongation was more in CS-TNPs as compared to TNPs which may be due to the presence of CS over TNPs creating a barrier for the drug release and prolonging the release of drug. The MRT of TNPs, HA-TNPs and CS-TNPs was found to be 8.8 folds, 7.9 folds and 7.9 folds higher respectively than pure TMZ which demonstrated long circulating properties of the nanoparticles (2). All obtained results can also be correlated with the metabolic pattern of TMZ *in vivo*. IV administered TMZ got quickly metabolized and degraded in physiological pH. However, when administered as nanoparticles (TNPs, HA-TNPs and CS-TNPs), they acted as a reservoir for TMZ, provided sustained release, decreased direct contact of drug with blood which

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reduced its metabolism and degradation, leading to prolonged MRT and higher AUC (6,7). From all these results, it may be concluded that TNPs, HA-TNPs and CS-TNPs enhanced the activity of TMZ by enhancing its half-life, bioavailability and circulating properties.



**Figure 8.1: In vivo pharmacokinetic study: Plasma concentration-time profile of TMZ, TNPs, HA-TNPs and CS-TNPs after intravenous injection in rats (n=6) and data is represented as mean  $\pm$  SD**

**Table 8.1: Pharmacokinetic parameters of TMZ, TNPs, HA-TNPs and CS-TNPs in rats (n=6). Data is represented as mean  $\pm$  SD**

Parameters	TMZ	TNP	HA-TNPs	CS-TNPs
$C_{\max}$ ( $\mu\text{g/ml}$ )	$4.91 \pm 0.11$	$2.27 \pm 0.12$	$3.22 \pm 0.1$	$3.47 \pm 0.15$
$T_{\max}$ (H)	0.5	3.0	6.0	6.0
AUC	$17.58 \pm 2.15$	$48.67 \pm 3.13$	$57.53 \pm 0.2$	$61.37 \pm 3.12$
$t_{1/2}$ (H)	$1.29 \pm 1.07$	$4.42 \pm 1.50$	$4.21 \pm 1.2$	$6.36 \pm 1.34$
MRT (H)	$2.33 \pm 1.25$	$20.50 \pm 2.10$	$18.30 \pm 1.5$	$18.45 \pm 2.14$

\* TMZ: temozolomide, TNPs: temozolomide loaded bovine serum albumin nanoparticles, HA-TNPs: hyaluronic acid modified TNPs, CS-TNPs: chondroitin sulphate modified TNPs,  $C_{\max}$ : maximum plasma concentration,  $T_{\max}$ : time at maximum plasma concentration, AUC: area-under-the-curve,  $t_{1/2}$ : half life, MRT: mean residence time.

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### 8.2.2            Biodistribution

The quantitative biodistribution study of TMZ, TNPs, HA-TNPs and CS-TNPs was performed to assess the passage of TMZ through the BBB and its distribution in various vital organs viz. brain, liver, heart, lungs, kidney and spleen after intravenous injection via tail veins. The obtained results are demonstrated in Figure 8.2. The distribution of TMZ in brain was approximately six folds ( $31.9 \pm 6.30 \mu\text{g/ml}$ ), eight folds ( $41.6 \pm 7.1 \mu\text{g/ml}$ ) and nine folds higher ( $43.5 \pm 6.87 \mu\text{g/ml}$ ) with TNPs, HA-TNPs and CS-TNPs respectively as compared to free TMZ ( $5.7 \pm 1.21 \mu\text{g/ml}$ ). This may be due to enhanced BBB permeability, passive targeting ability of TNPs and receptor mediated targeting along with passive targeting ability of HA-TNPs and CS-TNPs. When HA-TNPs and CS-TNPs were compared with TNPs, higher brain distribution of TMZ was observed which may be due to surface modification of TNPs with HA and CS. As mentioned earlier, CS also plays role in enhancing BBB permeation. The presence of CS over the nanoparticles facilitated the BBB crossing and enhance concentration of drug in the brain (8). The obtained results showed correlation with in vitro BBB permeation study of HA-TNPs and CS-TNPs, where permeation of TMZ was enhanced by three folds and two folds respectively. The results indicated enhanced permeation of drug through the tight junctions of the BBB when given as nanoparticles.

Further, the results revealed significant reduction in the distribution of drug to highly perfused organs when given as nanoparticles (TNPs, HA-TNPs and CS-TNPs). This may be attributed to surface modification of nanoparticles that prevented opsonization (2). After 24 h, TMZ released from TNPs, HA-TNPs and CS-TNPs were more or less similar in spleen and heart whereas in liver and lungs, distribution was significantly decreased as compared to pure TMZ. This may be due to reduced opsonization and decreased passive accumulation of drug in case of lungs (2) and due to hydrophilicity of nanoparticles in case of liver (9). As compared to TNPs, more accumulation of HA-TNPs and CS-TNPs in liver was observed which may be due to presence of hydrophilic polymer (HA/CS) over TNPs which enhanced their hydrophilicity. HA-TNPs and CS-TNPs showed much lesser accumulation of TMZ in lungs as compared to TMZ and TNPs. Distribution of HA-TNPs and CS-TNPs was high in kidney as compared to free TMZ

and TNPs which may be also due to more hydrophilicity of HA-TNPs and CS-TNPs than TMZ and TNPs (10,11).

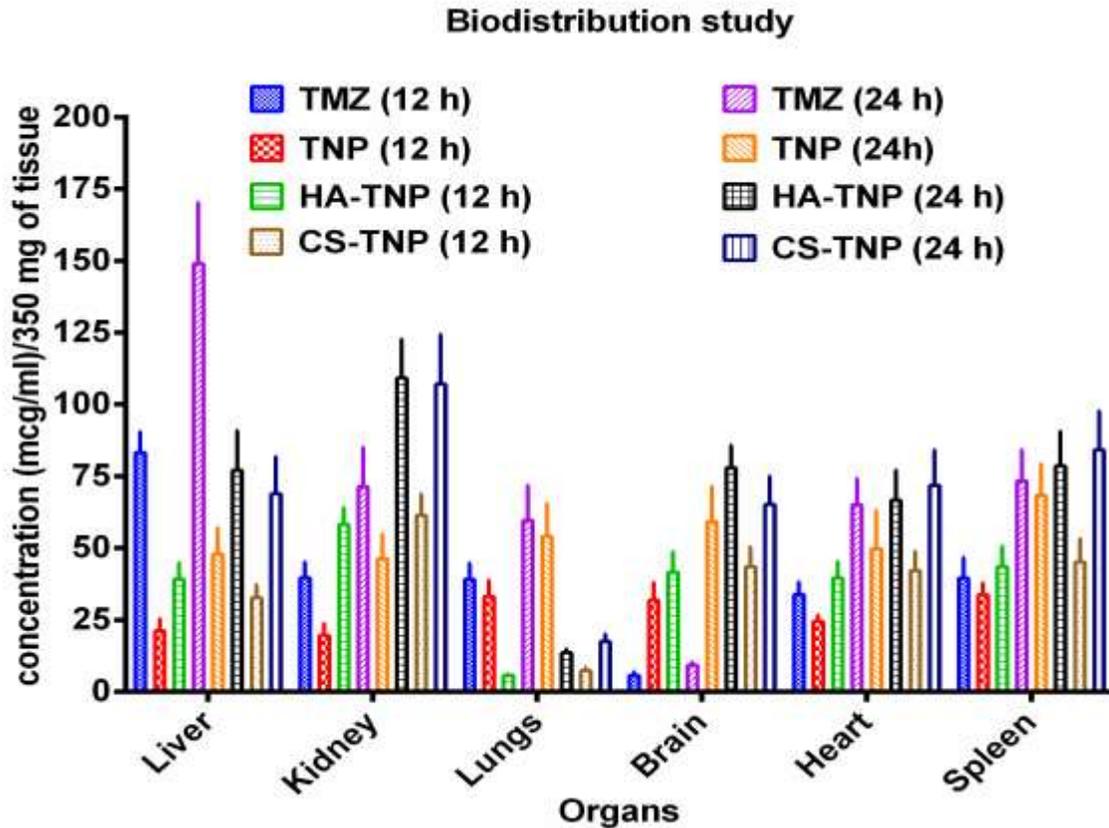


Figure 8.2: Biodistribution pattern of TMZ, TNPs, HA-TNPs and CS-TNPs

### 8.2.3 In vivo toxicity study

The in vivo toxicity study was performed to assess the safety potential of developed nanoparticles towards normal cells. Single dose toxicity method was used for demonstrating the in vivo toxicity of prepared TNPs, HA-TNPs and CS-TNPs as compared to pure TMZ in rats. In this study, neither rat death nor obvious abnormality was noticed after intravenous administration of TNPs, HA-TNPs and CS-TNPs (dose = 30 mg/kg) upto 7 days. The estimated biochemical parameters are summarized in Table 8.2. The results demonstrated no significant changes in the hematological parameters, including hemoglobin (HB), number of neutrophils (N), white blood cells (WBC), red blood cells (RBC), and platelets (PLT) after intravenous administration of

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nanoparticles. The suppression of immune cells (white blood cells, neutrophils, monocytes) was lesser in HA-TNPs and CS-TNPs treated rats as compared to TMZ and TNPs treated rats which may be due to sustained release of TMZ from HA-TNPs and CS-TNPs. The results also demonstrated no significant changes in the blood parameters (red blood cells, hemoglobin) and serum enzyme marker level (bilirubin, serum glutamic-oxaloacetic transaminase, serum glutamic pyruvic transaminase, alkaline phosphatase, albumin, globulin, blood urea nitrogen, creatinine) in TNPs, HA-TNPs and CS-TNPs treated rats and were found to be closely similar to non treated control rats. These results revealed minimal toxicity of drug towards normal cells after encapsulation in TNPs and CS-TNPs and indicated safe for administration. Similar studies were also reported by Kumari et al. 2017, where drug related toxicity was minimized after encapsulating in nanoparticles (11).

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**Table 8.2: Biochemical parameter estimation after i.v administration of nanoparticles after 7 days. . Data is represented as mean  $\pm$  SD (n=6)**

Parameters	Control	TMZ	TNPs	HA-TNPs	CS-TNPs
<b>HB%</b>	15.08 $\pm$ 0.42	14.29 $\pm$ 0.45	14.90 $\pm$ 0.54	15.10 $\pm$ 0.50	14.96 $\pm$ 0.35
<b>RBC *10<sup>3</sup>/cmm</b>	8.92 $\pm$ 0.67	8.17 $\pm$ 0.33	8.52 $\pm$ 0.41	8.55 $\pm$ 0.35	8.35 $\pm$ 0.47
<b>WBC*10<sup>3</sup>/cmm</b>	11.15 $\pm$ 1.24	10.35 $\pm$ 0.94	10.90 $\pm$ 1.03	10.90 $\pm$ 0.56	11.02 $\pm$ 0.64
<b>PLT*10<sup>5</sup>/cmm</b>	5.12 $\pm$ 0.43	4.95 $\pm$ 0.26	5.06 $\pm$ 0.77	5.05 $\pm$ 0.41	4.98 $\pm$ 0.39
<b>N%</b>	55.4 $\pm$ 4.55	54.86 $\pm$ 4.12	55.05 $\pm$ 3.98	55.08 $\pm$ 4.90	55.01 $\pm$ 5.72
<b>E%</b>	0.68 $\pm$ 0.05	0.65 $\pm$ 0.03	0.71 $\pm$ 0.04	0.69 $\pm$ 0.03	0.68 $\pm$ 0.05
<b>L%</b>	35.12 $\pm$ 3.47	34.12 $\pm$ 2.77	35.95 $\pm$ 2.17	34.75 $\pm$ 3.15	34.90 $\pm$ 3.75
<b>M%</b>	0.9 $\pm$ 0.03	0.88 $\pm$ 0.02	0.93 $\pm$ 0.02	0.89 $\pm$ 0.01	0.88 $\pm$ 0.02
<b>PCV%</b>	49.3 $\pm$ 4.66	48.7 $\pm$ 5.12	48.6 $\pm$ 4.99	49.21 $\pm$ 3.47	49.15 $\pm$ 5.88
<b>Bil (mg/dl)</b>	0.38 $\pm$ 0.01	0.36 $\pm$ 0.01	0.38 $\pm$ 0.01	0.38 $\pm$ 0.01	0.36 $\pm$ 0.02
<b>SGOT (IU/L)</b>	201.2 $\pm$ 23.54	197.5 $\pm$ 19.3	201.8 $\pm$ 21.5	202.7 $\pm$ 18.7	199.7 $\pm$ 24.1
<b>SGPT (IU/L)</b>	149.5 $\pm$ 21.80	147.3 $\pm$ 20.4	152.1 $\pm$ 19.6	152.7 $\pm$ 23.1	151.8 $\pm$ 19.0
<b>ALK (IU/L)</b>	135.7 $\pm$ 19.90	132.9 $\pm$ 18.6	133.6 $\pm$ 19.1	138.2 $\pm$ 18.7	137.9 $\pm$ 21.7
<b>PRO (g/dl)</b>	8.14 $\pm$ 1.00	7.92 $\pm$ 1.23	7.99 $\pm$ 1.06	8.11 $\pm$ 1.13	8.05 $\pm$ 1.19
<b>ALB (g/dl)</b>	3.26 $\pm$ 0.56	3.10 $\pm$ 0.43	3.15 $\pm$ 0.55	3.21 $\pm$ 0.44	3.21 $\pm$ 0.49
<b>GLB (g/dl)</b>	4.22 $\pm$ 0.33	4.17 $\pm$ 0.21	4.19 $\pm$ 0.21	4.24 $\pm$ 0.37	4.20 $\pm$ 0.41
<b>BUN (mg/dl)</b>	12.32 $\pm$ 1.12	12.11 $\pm$ 1.18	12.19 $\pm$ 1.90	12.36 $\pm$ 1.17	12.51 $\pm$ 1.92
<b>CREAT (mg/dl)</b>	1.38 $\pm$ 0.31	1.35 $\pm$ 0.33	1.35 $\pm$ 0.61	1.36 $\pm$ 0.37	1.36 $\pm$ 0.41

\* TMZ: temozolomide, TNPs: temozolomide loaded bovine serum albumin nanoparticles, HA-TNPs: Hyaluronic acid modified TNPs, CS-TNPs: chondroitin sulphate modified TNPs, HB = hemoglobin, RBC = red blood cells, WBC = white blood cells, PLT = platelet, N = neutrophils, E = eosinophils, L = lymphocytes, M = monocytes, PCV = packed cell volume, Bil = bilirubin, SGOT = serum glutamic-oxaloacetic transaminase, SGPT = serum glutamic pyruvic transaminase, ALK = alkaline phosphatase, ALB = albumin, GLB = globulin, BUN = blood urea nitrogen, CREAT = creatinine

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