Chapter 9
Radiolabelling and In vivo Biodistribution of Adefovir Dipivoxil formulations

Radiolabelling and In vivo biodistribution of Adefovir dipivoxil formulations

9.1 Introduction:

The objective of the present study was to examine the pharmacokinetics and biodistribution of ^{99m}Tc labeled Adefovir Dipivoxil formulations after oral administration to mice. The pharmacokinetic & biodistribution of two formulations in nanometer size range (Solid Lipid Nanoparticle and Nanosuspension) with conventional suspension was studied. Intravenous administration of conventional suspension was also carried out to determine the relative bioavailability values of Adefovir dipivoxil following oral administration in different formulations. Quality control test such as labelling efficiency (radiochemical purity) and stability of labeled complex in buffer, serum, Cysteine, Histidine and DTPA were performed prior to pharmacokinetic and biodistribution study. Another purpose of the present investigation was to compare the efficiency of SLN versus drug nanocrystals to enhance oral drug absorption of Adefovir dipivoxil.

9.2 Materials:

Diethylene triamine penta acetic acid (DTPA), cysteine, histidine, mice serum and stannous chloride dehydrate ($SnCl_2$. H_2O) were purchased from Sigma-Aldrich, Germany, Sodium pertechnetate from BARC (Bhabha Atomic Research Center, Mumbai, India), Instant thin layer chromatography ITLC-SG was purchased from Gelman science. Inc., Ann Arbor, MI.

9.3 Method:

9.3.1 Labeling Efficiency:

The radiochemical purity of ^{99m}Tc with Adefovir loaded Solid Lipid Nanoparticles (ADSLN), Adefovir Dipivoxil Nanosuspension (ANS) and Adefovir Dipivoxil Suspension (AMS) were estimated by paper chromatography (PC) and ascending instant thin layer chromatography (ITLC) using silica gel coated fiber sheets (Theobald AE., 1990). The ITLC and PC were performed using 0.9% saline as the mobile phase to determine the percentage of ^{99m}Tc, pertechnetate in the radiolabelled preparation. The amount of reduced technetium was determined by ITLC-SG using pyridine: acetic acid: water (3:5:1.5 v/v) as mobile phase. The strip was cut and the

radioactivity in each segment was determined in a well type gamma ray counter (Sodium iodide [Thalidium (Tl)] scintillation counter, Electronics Corporation of India Ltd., Mumbai). The reduced/hydrolysed (R/H) technetium along with the labeled complex remained at the point of application while both the free pertechnetate and the labeled complex moved away with the solvent front. By subtracting the radioactivity moved with the solvent front using saline from that using pyridine/acetic acid/water as a mixture, the net amount of 99mTc- formulation (SLN/NS/MS) was calculated.

9.3.2 Optimization of radiolabeling of Adefovir dipivoxil loaded SLN, NS and MS by direct labeling procedure:

The radiolabeling of formulations (ADSLN, ANS , AMS) were carried out using direct labeling procedure with ^{99m}Tc by simple reduction method using stannous chloride **(Arulsudar N., et al., 2003)**. Briefly, 1.0 ml of ^{99m}Tc in saline (2 mCi/ml) was added along with 0.1 ml of 0.5M bicarbonate buffer pH 9.0 followed by 0.1 ml of stannous chloride ($SnCl_2$) solution (1mg/ml) to the respective formulation. The labeling was carried out by mixing the reagents at ambient temperature for 10 to 15 minutes. The radiochemical purity of the labeled complex was estimated by ascending ITLC using 0.9% saline as developing solvent. Labeling procedure was standardized with respect to reagent concentrations and reaction parameters to achieve stable labeling in higher yields.

9.3.3 Stability Study of 99mTc-labeled Complexes:

Stability studies of radiolabelled complexes were carried out by incubating 0.1 ml of labeled preparation at different conditions similarly described in 6.5.

9.3.4 Pharmacokinetic and Biodistribution study:

These studies were conducted using the same protocol & procedures as per 6.6. Only in this case, each animal was administered with 0.5 mL of the ^{99m}Tc-labeled ADSLN, ANS and AMS (1.3 mg/kg) by oral gavage. Blood was withdrawn by cardiac puncture after different time intervals (1, 4, 8 and 24 hrs) and the mice were sacrificed by chloroform inhalation.

In a separate series of experiments, the ^{99m}Tc-labeled ADF in suspension (AMS) was administered intravenously (i.v.) via the tail vein in mice. For these studies, the formulations were made such that 26 µg of total ADF and 1µC of radioactivity was incorporated in 100µL of the injectable suspension formulations. At specific time

points (1, 4, 8 and 24 hrs), a group of three anesthetized mice were sacrificed by chloroform inhalation. Blood was withdrawn by cardiac puncture and radioactivity present in the whole blood was calculated as mentioned above.

9.3.5 Pharmacokinetic analysis:

The noncompartmental pharmacokinetic analysis was performed as per procedure in 6.7.

9.3.5.1 Data analysis:

The statistical significance of the differences between the formulations was tested by the one way ANOVA followed by Dunnets comparison test. All the values were reported as mean ± standard error of mean (S.E.M.) of three determinations.

9.4 Results and Discussion:

9.4.1 Stability of the ^{99m}Tc labeled complexes:

The radiolabeling of formulations (ADSLN, ANS, and AMS) were carried out using direct labeling procedure with ^{99m}Tc by simple reduction method using stannous chloride. Radiochemical purity and stability data of the labeled formulations were obtained by ITLC using 0.9% saline. These labeled complexes were tested for stability in saline and serum for 24 hrs. Table 9.1 shows the radiolabelling efficiency of ^{99m}Tc labeled Adefovir loaded formulations. The data demonstrates that the labeled complexes remained stable in- vitro in saline as well as serum upto 24 hrs. The labelling of all the three formulations provided in good yields and stability. Formulations were labeled with high efficiency of more than 80 %. The serum stability of these labeled complexes indicated their use as suitable markers for biodistribution study.

9.4.2 Cysteine, Histidine and DTPA challenge tests:

In vitro stability of radiolabeled complexes was determined by Cysteine, Histidine and DTPA challenge studies. High binding affinity of the labeled complexes was ascertained by incubating the labeled formulations with cysteine (0.1 M), histidine (0.1 M) and DTPA (50 and 100mM) (Table 9.2 and 9.3). Challenge test performed with DTPA in 50 mM and 100 mM concentration did not exhibit significant transchelation which was about 6-10 %. This observation confirmed the high strength and binding affinity of ^{99}Tc with formulation.

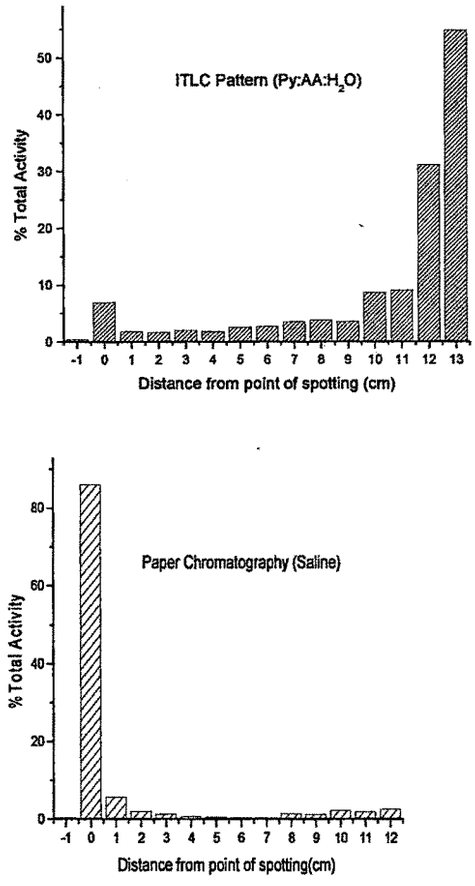


Fig 9.1. Chromatographic Characterization of ^{99m}Tc ADF Solid Lipid nanoparticles

Table 9.1: Stability of radiolabeled ADSLN, ANS and AMS formulation in saline (Room Temperature) and Serum (37°C).

	% Radiolabeling Efficiency					
	Saline			Serum		
Time	1 hrs	4 hrs	24 hrs	1 hrs	4 hrs	24 hrs
ADSLN	88.23	86.60	85.01	89.22	85.45	82.67
ANS	89.99	86.20	84.20	88.24	85.87	81.88
AMS	87.11	85.50	83.11	85.98	83.09	80.09

Table 9.2: Cysteine and Histidine challenge test of radiolabeled ADSLN, ANS and AMS formulation after 1 hr at 37°C.

Sample	% Transchelation	
	0.1 M Cysteine	0.1 M Histidine
ADSLN	7.98	6.98
ANS	6.87	7.67
AMS	8.98	8.75

Table 9.3: DTPA challenge test of radiolabeled ADSLN, ANS and AMS formulation for 2 hrs at 37°C

Conc. Of DTPA	% Transchelation		
	ADSLN	ANS	AMS
50 mM	5.09	7.77	6.59
100 mM	7.62	9.65	9.91

9.4.3 Pharmacokinetic study: Pharmacokinetic and Biodistribution study of ^{99m}Tc labeled Adefovir formulations i.e. ADSLN, ANS and AMS were investigated in healthy Swiss albino mice. Blood was obtained by cardiac puncture, weighed and the radioactivity present in the whole blood was calculated by keeping 7.3% of the body weight as total blood weight. Percentage radioactivity of injected dose per gram of organ was obtained which was converted to microgram per gram of organ.

The plasma concentration- time curve after oral and I.V. administration of AMS is shown in Fig. 9.2 and plasma concentration–time profile of three formulations AMS, ANS and ADSLN after oral administration is shown in Fig 9.3. Table 9.4 gives Mean plasma concentration (µg/gm) of AMS, ANS and ADSLN after oral administration. The Pharmacokinetic parameters after oral administration of AMS, ANS and ADSLN and Intravenous administration of AMS are summarized in Table 9.5.

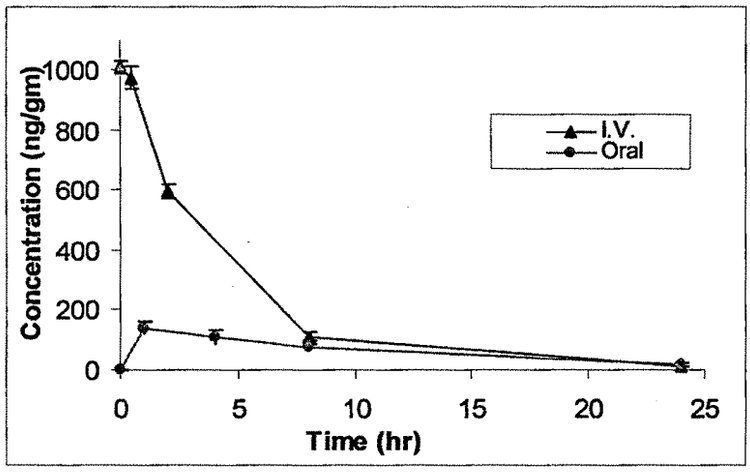


Fig. 9.2 Plasma concentration- time curve after oral and I.V. administration of Adefovir Microsuspension (AMS).

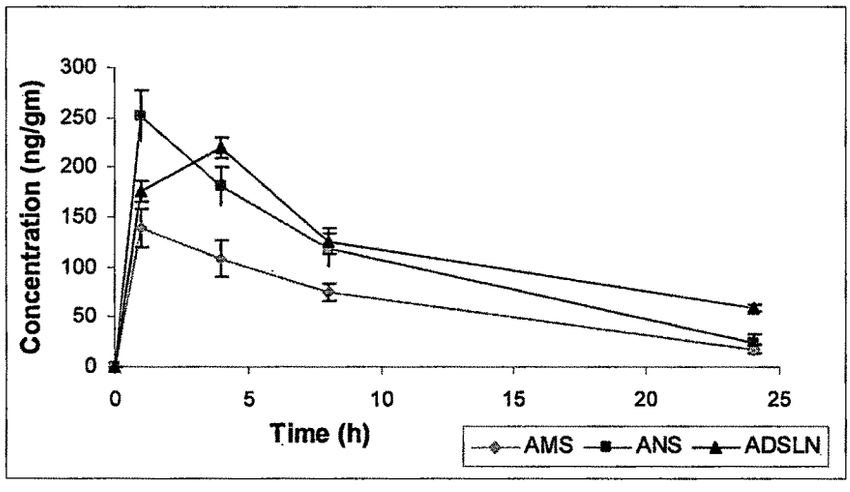


Fig 9.3 Plasma concentration time profile of AMS, ANS and ADSLN after oral administration.

Table 9.4 Mean plasma concentration (ng/gm) after oral administration of AMS, ANS and ADSLN.

Time	Concentration ± S.E.M. (ng/gm)		
	AMS	ANS	ADSLN
0	0	0	0
1	140± 16	252 ± 26	176 ± 10
4	109 ±18	181 ±19	220 ±11
8	75 ±9	118 ±16	126 ±13
24	18 ±4	25 ±6	59 ±7

Table 9.5 Pharmacokinetic parameters after oral administration of AMS, ANS and ADSLN and Intravenous administration of AMS

Parameters	Formulation			
	Adefovir suspension (control)		Adefovir Nanosuspension	Adefovir SLN
	I.V.	oral	oral	oral
Cmax (ng/g)	---	140±10.97	252±15.01*	176±5.77***
tmax (hr)	---	1	1	4
$t_{1/2}$ (h)	4.98±0.25	7.76±0.14	20.26±0.27*	12.76±0.31*
AUC (0 → t) ng.h/L	4533.5±81	1681±43.3	2068±34*	2826.5±23.23*
AUC (0 → inf) ng.h/L	5517±96.09	2299.5±55	3661.09±42.22*	4334.55±31*
Fr (%)	100	41.68	66.36	78.56
MRT (h)	4.05±1.34	10.45± 1.52	9.412± 2.01**	19.74± 1.24*
MAT (h)	----	6.40±1.3	5.36±1.8**	15.69±1.45*
Kel (hr^{-1})	0.246±0.07	0.095± 0.004	0.037± 0.004*	0.056±0.002*
VD (lit)	0.13± 0.04	0.82±0.09	0.46±0.07	0.82±0.06

Each value represents the mean ± S.D. of three determinations (n=3).

- Comparisons of ANS and ADSLN were made to AMS (control).
- * $P < 0.01$
- ** $P > 0.05$
- ***$P<0.05$
- Ns – non significant - $P > 0.05$

After oral administration, nanoparticulate formulations i.e. ADSLN and ANS exhibited higher plasma level concentration compared to AMS (control). The AUC $_{(0\rightarrow inf)}$ for the intravenous administration and oral suspension were about 5517±96 and 2299.5±55 ng.h/L respectively which was significantly different (*p<0.01*; ANOVA followed by Dunnett's multiple comparison test). Following oral administration of Adefovir nanosuspension (ANS) and Adefovir SLN (ADSLN) formulations to mice, the AUC $_{(0\rightarrow inf)}$ values were 3661.09±42.22 and 4334.55±31 ng.h/L respectively which were significantly different (*p<0.001*) from AUC obtained on oral administration of AMS.

The relative bioavailability for ANS and ADSLN were 66.36 % and 78.56 % respectively compared to 41.68 % bioavailability obtined after administration of AMS. It indicates improvement in bioavailability of Adefovir from nanoparticulate formulation than Microsuspension. Highest C_{max} (252±15.01µg/gm) amongst all tested formulations was observed with ANS followed by ADSLN (176±5.77 µg/gm) and AMS (252±15.01 µg/gm) .The statistical difference was more significant with ANS (P< 0.01) than ADSLN (P<0.05).

The average $T_{1/2}$ values were 20.26±0.27, 12.76±0.31h for ANS and ADSLN formulations, respectively, as compared to 4.98± 0.25 h and 7.76± 0.14 h following administration of I.V.injection and oral administration of AMS. There was stastically significant difference in $T_{1/2}$ value of ANS & ADSLN compared to AMS (P< 0.01). Increase in biological half life signifies increased time of ADF in plasma from nanoparticulate formulation compared to AMS.

The sustained-release characteristic of the ADSLN was reflected in the MRT in the body. MRT was considerably increased following administration of the ADSLN as compared to ANS. The average MRT after oral administration of ADSLN, ANS and AMS were 19.74± 1.24, 9.412± 2.01 and 10.45± 1.52 h, respectively, as compared to 4.05±1.34 h after I.V. administration. MRT value of ANS was not found to be statistically differenct than oral ANS (P>0.05) while value of ADSLN was statistically different (P<0.01). This may be due to prolonged release of ADF from ADSLN as observed in *in vitro* release compared to ANS formulation. Also the bioadhesive property of SLN helps to improve residence time in gut in turn improving absorption of SLN over a period of time (Irache et al 1998).

9.4.4 Biodistribution Study:

In case of ^{99m}Tc labeled AMS, ANS and ADSLN, **Liver, Intestine, kidney and Stomach** accumulated major portion of the administered radioactivity. **Liver** is one of the major organs of reticuloendothelial system (RES) which are known to accumulate and metabolize nanoparticles (Arien A. et al 2006). The biodistribution data (Table 9.6) reveals higher initial rapid uptake by liver, which was 2990, 2488, 1746 ng/gm for AMS, ANS and ADSLN after 1 hour of oral administration respectively. It can be observed that uptake was faster for AMS than nanoparticles. After 24 hours, 226.5, 645 and 1200 ng/gm of AMS, ANS and ADSLN was observed, indicating rapid metabolism of AMS compared to ANS and ADSLN. Also, higher concentration of ADSLN observed in liver after 24 hours is significant point as Adefovir as is indicated for the treatment of chronic Hepatitis B infection.

The radioactivity in **stomach** was found to decrease rapidly in case of ANS and ADSLN than AMS. In case of AMS, stomach activity was decreased from 2206 to 746 ng/gm compared to ANS (2594 to 162 ng/gm) and ADSLN (3211 to 370 ng/gm) after 1 hr to 24 hrs. This suggests rapid absorption of ADSLN and ANS than AMS from stomach to intestine. It is also evident from relatively higher concentration of ANS and ADSLN in blood compared to AMS.

There was difference in time to reach maximum radioactivity in **intestine.** AMS showed maximum radioactivity after 4 hrs, ANS - after 1 hr and ADSLN – after 8 hour. After 24 hours, highest radioactivity was observed for ADSLN (1129.2 ng/gm) followed by ANS (687 ng/gm). The possible explanation is that nanoparticles would allow a more intimate contact with the absorptive cells in the gut due to their bioadhesive properties (Irache et al 1998).

Kidney also showed higher uptake of labeled complexes in mice compared to other organs. After 1 hour administration, 2316 ng/gm of AMS was observed while 1889 and 1887 ng/gm of ADF was observed after administration of ANS and ADSLN. Although, there was no statistically significant difference between ANS and ADSLN after 24 hours of administration ($p>0.05$ ANOVA followed by Dunnett's multiple comparison test). It was also observed that compared to other organs (heart, lungs and spleen) significant amount of radioacitivity was observed in kidney after 24 hours. Radioactivity was more for AMS (3186 ng/gm) than ANS (2900 ng/g) and ADSLN (2064.4 ng/gm).

Table 9.6: Biodistribution of ^{99m}Tc labeled AMS, ANS and ADSLN in mice

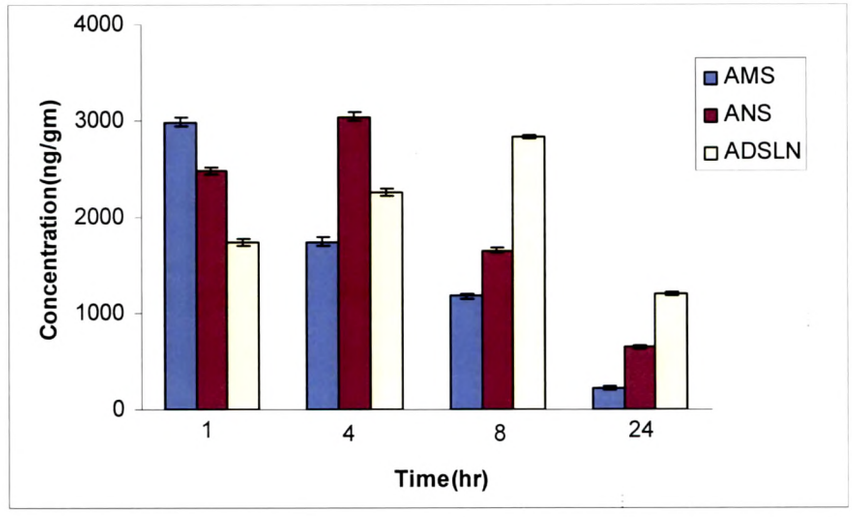
Organs	ng /gram of organ ± S.D.											
	1 Hrs			4 Hrs			8 Hrs			24 Hrs		
	AMS	ANS	ADSLN	AMS	ANS	ADSLN	AMS	ANS	ADSLN	AMS	ANS	ADSLN
Blood	140 ±16	252±26	176±10	109 ±18	181 ±19	220 ±11	75 ±9	118 ±16	126 ±13	18 ±4	25 ±6	59 ±7
Liver	2990± 49	2488 ± 39	1746± 27	1750± 38	3043± 41	2266± 26	1176± 21	1650± 19	2833± 23	226.5± 14	645± 17	1200± 18
Ints +GB	1846 ±43	2556 ± 41	1733 ± 34	2109 ± 37	1690 ± 31	2021±25	1516 ± 26	1777 ±25	2678 ±31	144± 15	687 ±17	1129.2 ± 13
Kidney	2316 ± 31	1889 ±22	1887 ±21	3609 ± 41	3222 ±38	2611 ±28	4843 ±35	4117 ±43	3645 ± 35	3186 ±22	2900 ±27	2064.6±20
Stomach	2206 ± 37	2594 ±31	3211 ±24	1900 ±25	1879 ±29	2109 ±18	1333 ±21	998 ± 25	1190 ±15	746 ±26	162 ±16	370 ± 11
Heart	34 ± 7	84 ±8	53 ±8	63 ±9	58 ±7	67 ±5	41 ±7	39 ±6	44 ±5	11 ±3	18 ±3	24 ± 5
Lungs	102 ± 24	176 ± 10	156 ± 11	143 ± 21	197 ± 9	214 ±9	86 ±16	156 ±13	177 ± 11	22 ± 5	79 ±7	101 ±8
Spleen	47 ± 9	93 ±11	183 ±19	77 ±13	129 ± 15	161 ±10	39 ± 6	52 ±5	117 ±14	11 ±3	13 ±3	45 ±4

The values represented here are the mean of three values with Standard Deviation (S.D.). Radioactivity is expressed as nanogram of administered dose per gram of tissue or organ.

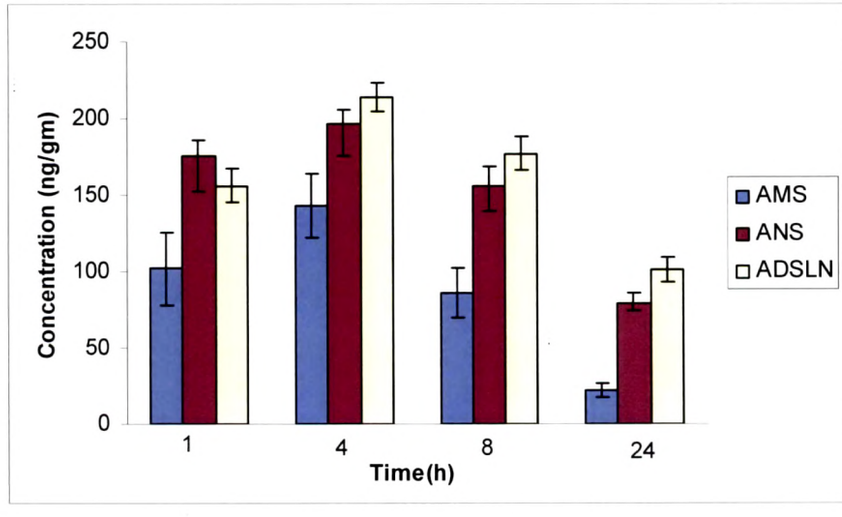
It is important to note that the stomach and intestines of the rats are cleaned of all food or waste material and thus the levels measured for these organs correspond to the levels of the actual tissue. No significant radioactivity was found in heart and lungs. In case of lungs, maximum radioactivity was observed for ANS (197 ng/gm) and ADSLN (214 ng/gm) followed by AMS (143 ng/gm) after 4 hrs.

Fig 9.4 Concentration time profile of ^{99m}Tc labeled AMS, ANS and ADSLN in different organs

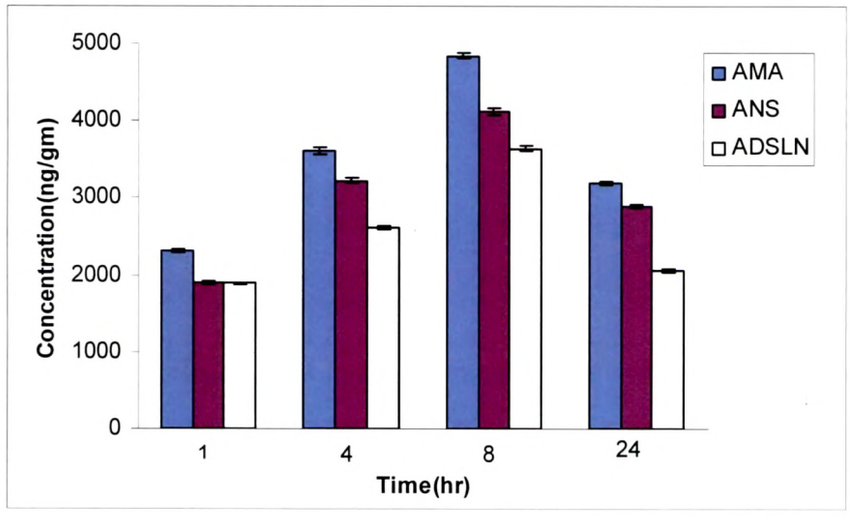
(a) Liver



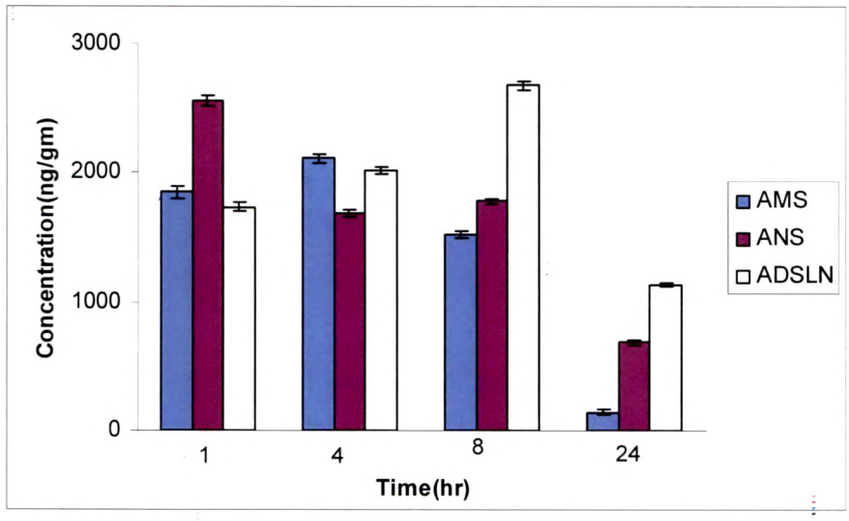
(b) Lungs



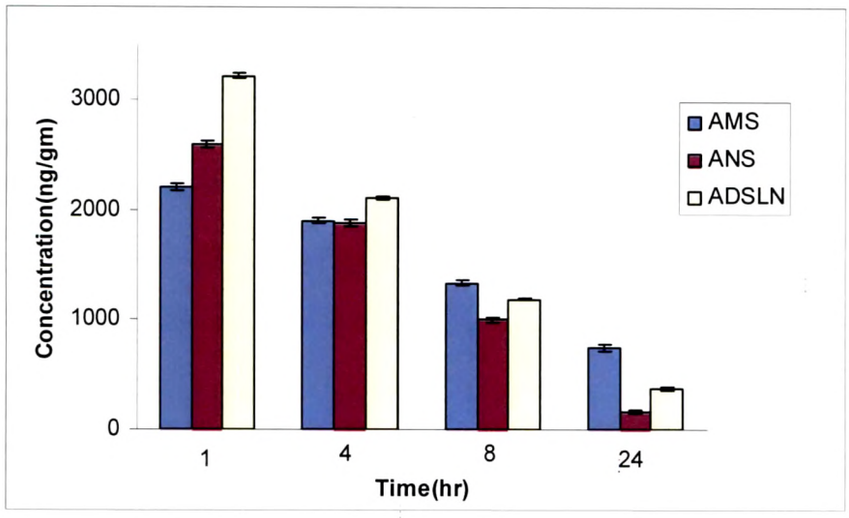
(C) Kidney



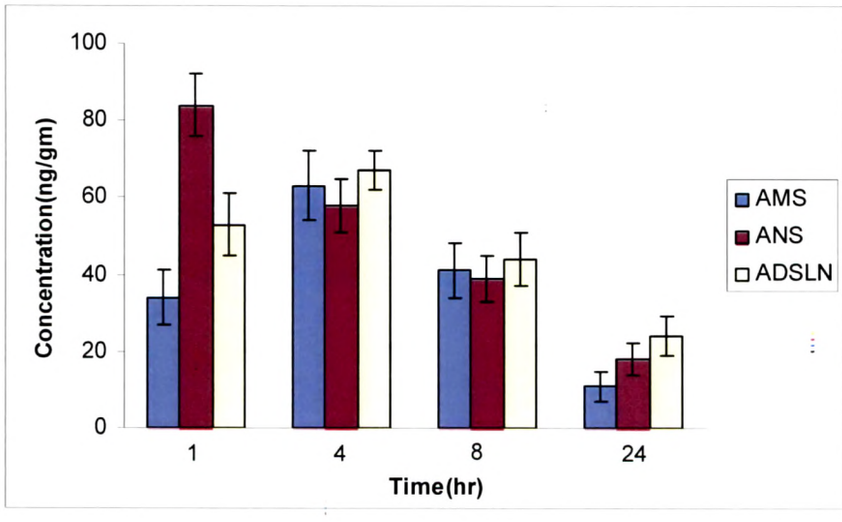
(d) Intestine



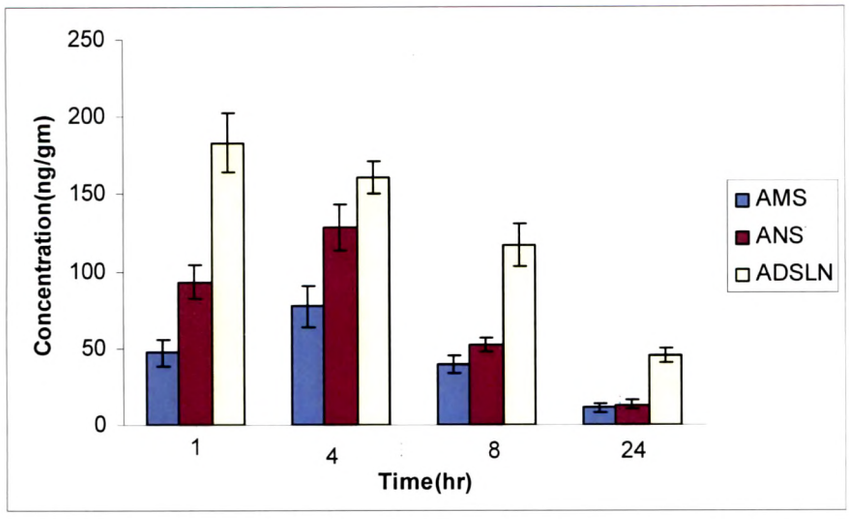
(e) Stomach



(f) Heart



(g) Spleen



Conclusion:

In the present study, biodistribution of Adefovir Dipivoxil was studied following oral administration. Three formulations were tested: a nanosuspension (ANS), one solid lipid nanoparticle preparation (ADSLN) and one suspension in micron size (AMS). Thus, both colloidal drug delivery systems (ANS and ADSLN) have shown increase in bioavailability compared to conventional suspension or microsuspension (AMS). Amongst colloidal drug delivery systems, SLN showed prolonged residence time in blood compared to ANS. Significant difference was observed in Cmax and AUC (0 → inf) between ANS, ADSLN compared to AMS. Thus nanoparticulate formulations i.e. SLN and NS hold more promise than conventional suspension in order to improve bioavailability of drugs.

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