CHAPTER 10

RADIOLABELING OF LIPOSOMES

10.1 INTRODUCTION

The methods employed to label liposomes include entrapment of the radiolabel in the aqueous compartment, attachment of the label to the lipid components prior to liposome formulation, and the addition of label after their manufacture. The biodistribution of liposomes administered in vascular or extra vascular spaces might be studied by the administration of radiolabeled liposomes and followed by scintigraphic imaging. Radiolabeled liposomes have been successfully used to monitor pharmacodynamic changes of liposomes and image tumors, abscesses, ischemic and infracted regions.

Liposomes were radiolabeled using various isotopes such as gallium-67 (Ogihara et. al., 1986), Indium-111 (Presne et. al., 1989) and Technetium-99m (^{99m}Tc) (Barratt et. al., 1984). The easiest and most commonly used isotope for labeling liposomes is ^{99m}Tc because of its unique properties of short half life, simple method of preparation, rapid and stable labeling (Saha, 1993).

Nearly 80% of all radiopharmaceuticals used in nuclear medicine are ^{99m}Tc-labelled compounds. The reason for such a predominant position of ^{99m}Tc in clinical use is its extremely favourable physical and radiation characteristics. The 6-hr physical half-life and the little amount of electron emission permit the administration of millicurie amounts of ^{99m}Tc radioactivity without significant radiation dose to the patient. In addition, the monochromatic 140 keV photons are readily collimated to give images of superior spatial resolution. Furthermore, ^{99m}Tc is readily available in a sterile, pyrogen free and carrier free state from ^{99}Mo-^{99m}Tc generators.

The use of radiolabels has proven quite useful in following the fate of liposomes *in vivo* and as diagnostic tools in nuclear medicine. The methods employed to label liposomes include entrapment of the radiolabel in the aqueous compartment, attachment of the label to the lipid components prior to liposome formulation, and the addition of label after their manufacture (Richardson et al., 1978). Radiolabeled liposomes have been successfully used to monitor pharmacodynamic changes of liposomes and image tumors, abscesses, ischemic and infracted regions (Richardson et al., 1978). ^{99m}Tc is

the best candidate for imaging studies due to its short half life, pure photon emitter and suitable energy.

10.2 CHEMISTRY OF TECHNETIUM

Technetium is a transition metal of silvery grey colour belonging to group VIIB (Mn, Tc and Re) and has the atomic number 43. No stable isotope of technetium exists in nature. The ground state ^{99}Tc has a half-life of 2.1 x 10^5 years. The electronic structure of the neutral technetium atom is $1s^22s^22p^63s^23p^63d^{10}4s^24p^64d^65s^1$. Technetium can exist in eight oxidation states namely, 1- to 7+, which result from the loss of a given number of electrons from the 4d and 5s orbitals or gain of an electron to the 4d orbital. The stability of these oxidation states depends on the type of ligands and chemical environment. The 7+ and 4+ states are the most stable and are represented in oxides, sulphides, halides and pertechnetates. The lower oxidation states 1-, 1+, 2+ and 3+, are normally stabilized by complexation with ligands. For example, Tc $^{1+}$, complexed with six isonitrile groups in ^{99m}Tc-sestamibi. Otherwise they are oxidised to 4+ state and finally to the 7+ state (Saha, 1993).

10.3 REDUCTION OF $^{99m}TcO_4^-$

The chemical form of ^{99m}Tc available from the Molybdenum generator is sodium pertechnetate (^{99m}Tc-$NaTcO_4$). The pertechnetate ion, $^{99m}TcO_4^-$, having the oxidation state 7+ for ^{99m}Tc, resembles the permanganate ion, MnO_4^-, and the perrhenate ion, ReO_4^- Chemically, $^{99m}TcO_4^-$ is a rather non-reactive species and does not label any compound by direct addition. In ^{99m}Tc- labeling of many compounds, prior reduction of ^{99m}Tc from 7+ state to a lower oxidation state is required (Saha, 1993). Various reducing systems that have been used are stannous chloride ($SnCl_2.2H_2O$), stannous citrate, stannous tartrate, concentrated HCl, sodium borohydride ($NaBH_4$), dithionite and ferrous sulphate. Among these, stannous chloride is the most commonly used reducing agent in acidic medium in most preparations of ^{99m}Tc-labelled compounds.

10.4 LABELING WITH REDUCED TECHNETIUM

The reduced ^{99m}Tc species are chemically reactive and combine with a wide variety of compounds, which usually donates lone pair of electrons to form

coordinate covalent bonds with ^{99m}Tc. Compounds bearing chemical groups such as $-COO^-$, $-OH^-$, $-NH_2$ and –SH are eligible for labeling with technetium.

10.5 HYDROLYSIS OF REDUCED TECHNETIUM AND TIN

There is a possibility that reduced ^{99m}Tc may undergo hydrolysis in aqueous solution. In this case, the reduced ^{99m}Tc reacts with water to form various hydrolysed species depending on the pH, duration of hydrolysis and presence of other agents. Some species of this category are $^{99m}TcO_2$, $^{99m}Tc^{2+}$ and $^{99m}TcOOH^+$. This hydrolysis competes with the chelation process of the desired compound and this reduces the yield of the ^{99m}Tc-chelate.

The use of stannous chloride has a disadvantage in that it also readily undergoes hydrolysis in aqueous solution at approximately pH 6 to 7 and forms insoluble colloids. These colloids bind to reduced ^{99m}Tc and thus compromise the labeling yield. To prevent this colloid formation, an acid is added to prevent the hydrolysis of Sn^{2+} before the reduction of technetium.

10.6 MATERIALS

Diethylene triamine penta acetic acid (DTPA) and stannous chloride dehydrate ($SnCl_2.2H_2O$) were purchased from Sigma Chemical Co., St.Louis, M.O.; Sodium pertechnetate separated from Molybdenum-99 by solvent extraction method was procured from Regional center for Radiopharmaceutical division (Northern Region), Board of Radiation and Isotope Technology, Delhi, India.

10.7 RADIOLABELING OF CYCLOSPORINE, LEUPROLIDE ACETATE, DNA AND THEIR LIPOSOMAL FORMULATIONS

The radiolabeling of the Cyclosporine, Leuprolide acetate, DNA and their liposomal formulations with reduced ^{99m}Tc were carried out as per the procedure given below.

10.7.1 LABELING EFFICIENCY

The radiochemical purity of ^{99m}Tc with the drugs and its liposomal formulations was estimated by ascending instant thin layer chromatography (ITLC) using silica gel coated fiber sheets (Gelman Sciences. Inc., Ann Arbor, MI). The ITLC was performed using 100 % acetone or 0.9 % saline as the

mobile phase. 2-3 µl of the radiolabeled complex was applied at a point 1 cm from one end of an ITLC-SG strip. The strip was developed in acetone or 0.9 % saline and the solvent front was allowed to reach 8 cm from the origin. The strip was cut into two halves and the radioactivity in each segment was determined in a well type gamma ray counter (Gamma ray spectrophotometer, Type GRS23C, Electronics corporation of India ltd., Mumbai). The free pertechnetate which moved with the solvent (R_f = 0.9) was determined. The reduced / hydrolysed (R/H) technetium along with the labeled complex remained at the point of application. The amount of reduced / hydrolysed ^{99m}Tc (radiocolloids) was determined using pyridine: acetic acid: water (3: 5: 1.5 v/v) as mobile phase. The R/H ^{99m}Tc remained at the point of application while both the free pertechnetate and the labeled complex moved away with the solvent front. By subtracting the activity moved with the solvent front using either acetone or saline from that using pyridine: acetic acid: water as a mixture, the net amount of ^{99m}Tc- liposome was calculated.

10.7.2 STABILITY STUDY OF ^{99M}TC- LABELED COMPLEX

The stability study of radiolabeled complex was determined *in vitro* using 0.9 % sodium chloride and serum by ascending thin layer chromatography (Chauhan *et. al.,* 1993). The complex (0.1 ml) was mixed with 1.9 ml of normal saline or rabbit serum and incubated at 37°C. ITLC was performed at different time intervals to assess the stability of the complex. Any increase in pertechnetate percentage was considered as the degree of degradation of the labeled complex.

10.7.3 DTPA CHALLENGING TEST

The binding affinity of the labeled complexes was confirmed by transchelation using DTPA. The stability of the complexes was examined by challenging with DTPA at different concentrations. The DTPA challenge assay involved incubation of the labeled complex with different concentrations of transchelator (25 –100 mM) at room temperature for a period of 1h. The effect of DTPA on labeling efficiency was measured on ITLC-SG using normal saline (Eckelman et. al., 1989) as the mobile phase which allowed the separation of free pertechnetate and DTPA- complex

0.8-1.0) from the ^{99m}Tc-liposome complex which remained at the point of application. ($R_f = 0$).

10.7.4 OPTIMIZATION OF RADIOLABELING OF CYCLOSPORINE AND ITS LIPOSOMAL FORMULATIONS

The cyclosporine and its liposomes were labeled with ^{99m}Technetium (^{99m}Tc) by direct labeling method as described earlier (Richardson et al., 1977). The radiolabeling of cyclosporine and its liposomes (CsA, CPL, CNL and CL) were done with Technetium-99m (^{99m}Tc) by simple reduction method as described earlier (Richardson et. al., 1977). The pertechnetate used for the study was first reduced to its lower valency state using stannous chloride dihydrate and then pH was adjusted to neutral before mixing with the leuprolide/liposome suspension. The radiolabeling was optimized by taking three factors into account. i.e. pH of the complex, incubation time and stannous chloride dihydrate concentration. The pH of the labeled complex was increased from 5 to 8 and its effect on labeling efficiency was studied. The radiolabeled complexes were incubated for various time periods and the effect of incubation time on labeling efficiency was determined keeping other variables constant. The effect of $SnCl_2.2H_2O$ concentration on the labeling efficiency was also studied to obtain the optimum concentration needed for maximum labeling. The radiolabeling procedure for cyclosporine and its liposome formulations is given below.

Briefly, 1 ml of ^{99m}Tc (2 mCi / ml) was mixed with 0.1 ml of stannous chloride solution (1mg / ml) and the pH was adjusted to seven using 0.5M sodium bicarbonate solution. To this mixture, 1 ml of cyclosporine solution (cyclosporine prepared in cremophor solution) (1mg / ml) or 1 ml of liposome suspension (lipid concentration – 15–20mg / ml) was added and incubated for 15 minutes for free drug and liposomes respectively at room temperature. The quality control was performed as per the method described earlier (Theobald, 1990). The effect of pH on labeling efficiency of ^{99m}Tc-cyclosporine/liposomes is given in table 10.1 and figure 10.1. The radiolabeled complex was incubated for various time periods and the effect of labeling on incubation time was determined keeping other variables constant as shown in table 10.2 and figure 10.2. The effect of stannous chloride concentration on the labeling efficiency was also studied to obtain the

optimum concentration needed for maximum labeling as shown in table 10.3. The in vitro stability studies of the labeled complexes were carried out in saline and in serum and the results are shown in table 10.4 and table 10.5.

Table 10.1 Effect of pH on radiolabeling efficiency of cyclosporine and its liposomal formulations

pH	Percent Radiolabeled (± S.E.M)			
	CsA	CPL	CNL	CL
5.0	85.35±0.42	84.26±0.99	86.42±0.46	85.46±0.79
5.5	87.5±0.35	89.67±0.46	89.23±0.48	88.72±0.89
6.0	92.4±0.23	93.6±0.56	95.4±0.58	91.34±0.95
6.5	99.17±0.36	97.42±0.69	99.31±0.78	96.9±0.65
7.0	96.35±0.33	98.23±0.58	98.7±0.88	97.3±0.86
7.5	90.6±0.43	90.17±0.98	91.6±0.98	88.78±0.76
8.0	85.36±0.62	85.54±0.36	86.23±0.77	84.13±0.39

Figure 10.1 Effect of pH on radiolabeling efficiency of cyclosporine and its liposomal formulations

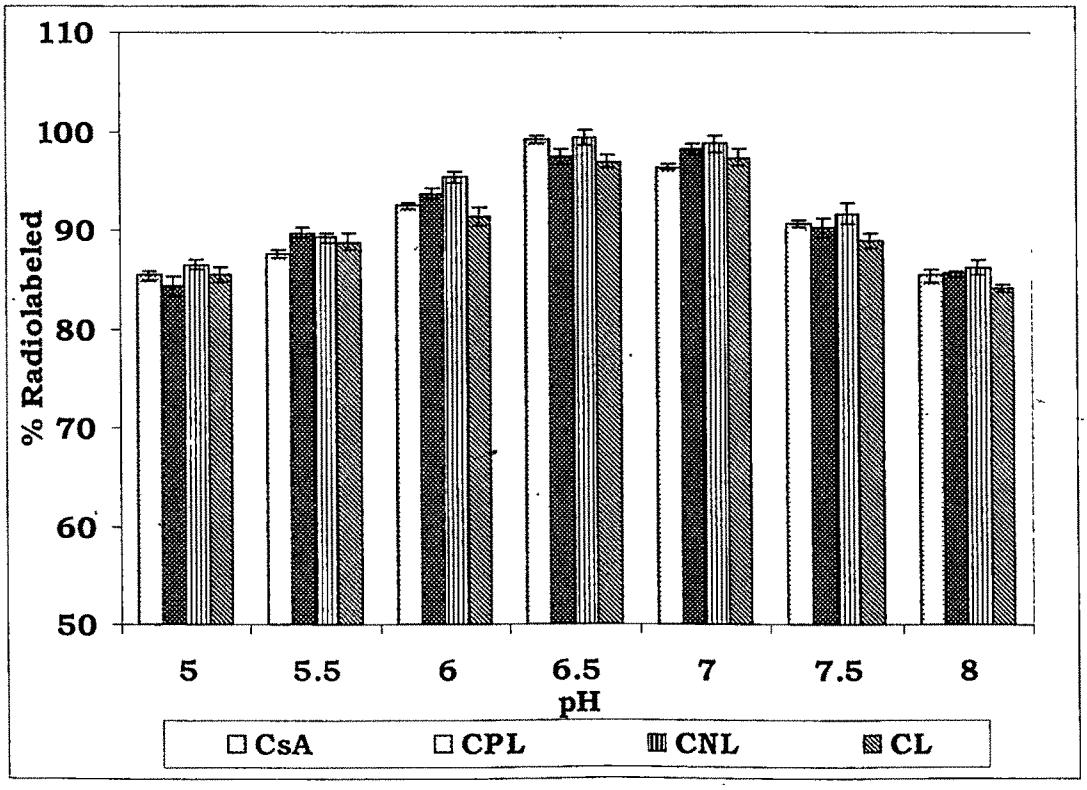


Table 10.2 Effect of incubation time on radiolabeling efficiency of cyclosporine and its liposomal formulations

Time of incubation (min)	% Radiolabeled			
	CsA	CPL	CNL	CL
0	92.49±1.26	92.5±1.02	93.6±0.99	90.8±0.65
5	98.54±1.32	94.3±0.77	97.23±0.65	96.1±0.68
15	99.17±0.99	97.5±1.06	98.9±1.02	96.6±1.23
20	98.29±0.89	95.6±1.11	97.79±1.36	96.1±1.22
30	98.8±1.56	95.9±0.94	98.86±1.78	97.5±0.98
40	98.54±1.23	96.4±0.85	98.23±0.63	97.2±1.46

Figure 10.2 Effect of incubation time on radiolabeling efficiency of cyclosporine and its liposomal formulations

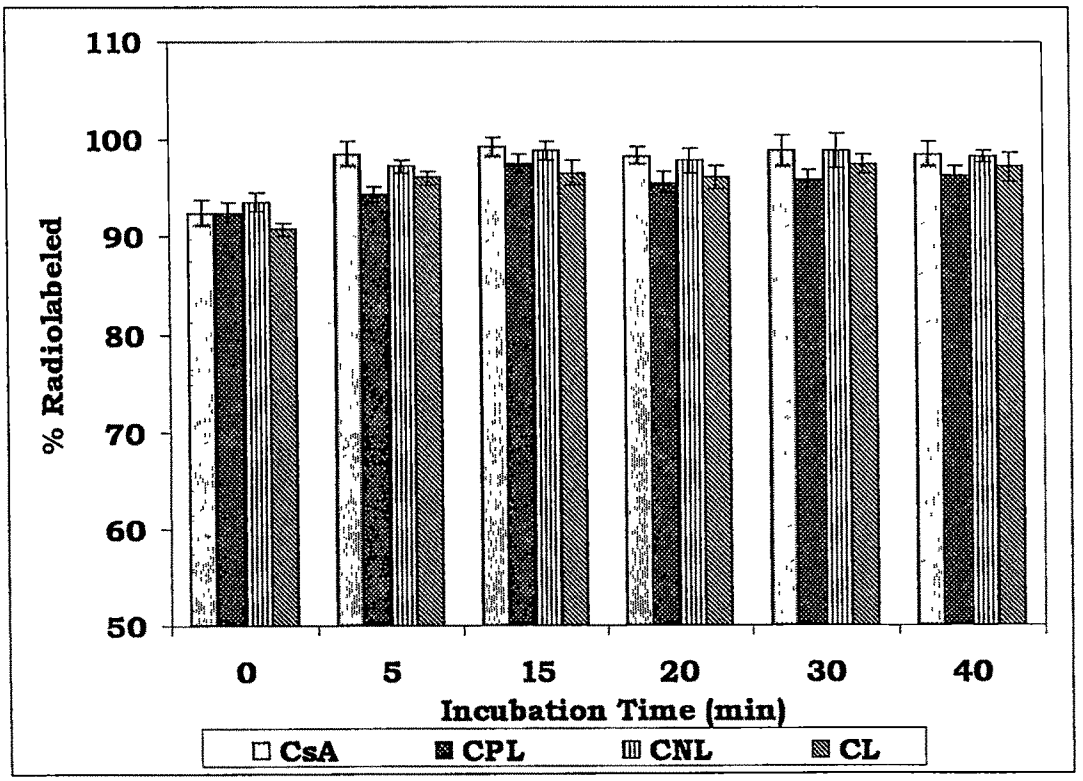


Table 10.3 Effect of $SnCl_2.2H_2O$ concentration on radiolabeling efficiency of cyclosporine and its liposomal formulations

$Sncl_2.2H_2O$ µg/ml	CsA			CPL			CNL			CL		
	% Bound	% colloids	% free	% Bound	% colloids	% free	% Bound	% colloids	% free	% Bound	% colloids	% free
25	85.23	0.5	14.27	85.62	0.12	14.26	83.26	0.12	16.62	82.64	2.23	15.53
50	87.68	0.56	11.76	90.23	0.5	9.27	89.52	0.5	10.98	90.68	1.86	7.46
100	99.17	0.78	0.05	97.42	0.56	3.14	99.31	0.56	0.13	97.1	1.28	1.62
150	97.23	2.57	0.2	93.62	3.52	2.86	96.09	3.52	0.39	96.12	2.86	1.02
200	93.59	6.23	0.18	93.08	6.35	0.57	93.43	6.35	0.22	92.08	7.56	0.36

Table 10.4 Stability studies of radiolabeled cyclosporine and its liposomal formulations in saline

	% Radiolabeling Efficiency						
	15 min	**30 min**	**1 h**	**2 h**	**4 h**	**6 h**	**24 h**
CsA	98.90	98.54	98.29	98.8	97.71	97.2	96.70
CPL	97.5	97.3	95.6	95.9	95.9	95.6	91.6
CNL	97.6	97.2	97.8	98.9	98.6	98.2	98.5
CL	96.9	96.4	97.5	96.15	97.14	97.02	93.13

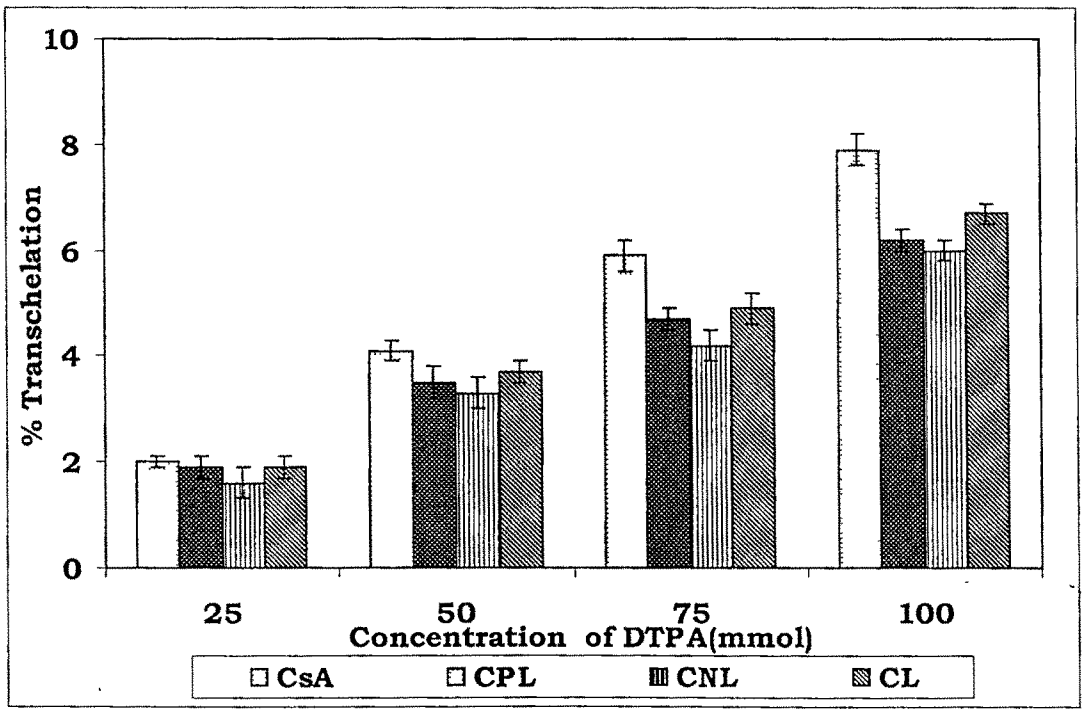
Table 10.5 Stability studies of radiolabeled cyclosporine and its liposomal formulations in serum

	% Radiolabeling Efficiency						
	15 min	**30 min**	**1 h**	**2 h**	**4 h**	**6 h**	**24 h**
CsA	97.23	97.03	97.26	95.23	95.20	95.4	95.02
CPL	97.62	97.23	97.09	96.02	96.09	96.15	96.12
CNL	97.50	97.21	96.72	95.20	94.80	94.79	94.81
CL	96.23	96.19	96.13	95.23	95.14	95.06	95.80

Table 10.6 DTPA challenging test of cyclosporine and its liposomal formulations

Conc. of DTPA (mM)	% Transchelation			
	CsA	CPL	CNL	CL
25	2.0±0.1	1.9±0.2	1.6±0.3	1.9±0.2
50	4.1±0.2	3.5±0.2	3.3±0.3	3.7±0.2
75	5.9±0.3	4.7±0.2	4.2±0.3	4.8±0.3
100	7.9±0.3	6.2±0.2	6.0±0.2	6.7±0.2

Figure 10.3 DTPA challenging test of cyclosporine and its liposomal formulations



10.7.5 OPTIMIZATION OF RADIOLABELING OF LEUPROLIDE AND ITS LIPOSOMAL FORMULATIONS

The radiolabeling of leuprolide acetate and its liposomes (LL, SLL5000-CC-PE, and SLL2000-CC-PE) were done with Technetium-99m (^{99m}Tc) by simple reduction method as described earlier (Richardson et. al., 1977). The pertechnetate used for the study was first reduced to its lower valency state using stannous chloride dihydrate and then pH was adjusted to neutral before mixing with the leuprolide/liposome suspension. The radiolabeling was optimized by taking three factors into account. i.e. pH of the complex, incubation time and stannous chloride dihydrate concentration. The pH of the labeled complex was increased from 5 to 8 and its effect on labeling efficiency was studied. The radiolabeled complexes were incubated for various time periods and the effect of incubation time on labeling efficiency was determined keeping other variables constant. The effect of $SnCl_2.2H_2O$ concentration on the labeling efficiency was also studied to obtain the optimum concentration needed for maximum labeling. The radiolabeling procedure for leuprolide and its liposome formulations is given below.

1ml of ^{99m}Tc pertechnetate (2mCi/ml) was mixed with specific amount of stannous chloride solution (1mg/ml) and the pH was adjusted suitably using sodium bicarbonate solution. To this mixture, 1ml of leuprolide acetate solution (500µg/ml) or 1ml of liposome suspension containing 500µg of drug was added and incubated for specific time period at room temperature. The radiochemical purity of labeled complex was estimated by ascending instant thin layer chromatography using 100% acetone or 0.9% sodium chloride as developing solvents (Theobald, 1990).

Table 10.7 Effect of pH on radiolabeling efficiency of leuprolide acetate and its liposomal formulations

pH	Percent Radiolabeled (± S.E.M)			
	Leuprolide	**LL**	**SLL5000**	**SLL2000**
5	70.56±0.56	78.62±0.95	74.23±1.23	76.86±0.94
5.5	72.43±0.63	80.23±0.33	82.62±0.89	80.18±0.87
6	89.26±0.36	86.13±0.65	85.68±0.68	87.23±0.76
6.5	96.93±0.39	98.18±0.94	97.23±0.96	97.68±1.23
7	96.56±0.68	98.9±0.64	98.9±0.84	98.3±1.08
7.5	90.26±0.99	91.52±0.88	92.62±1.26	89.68±1.23
8	85.68±1.02	86.99±1.03	84.56±1.05	83.26±1.02

Figure 10.4 Effect of pH on radiolabeling efficiency of leuprolide and its liposomal formulations

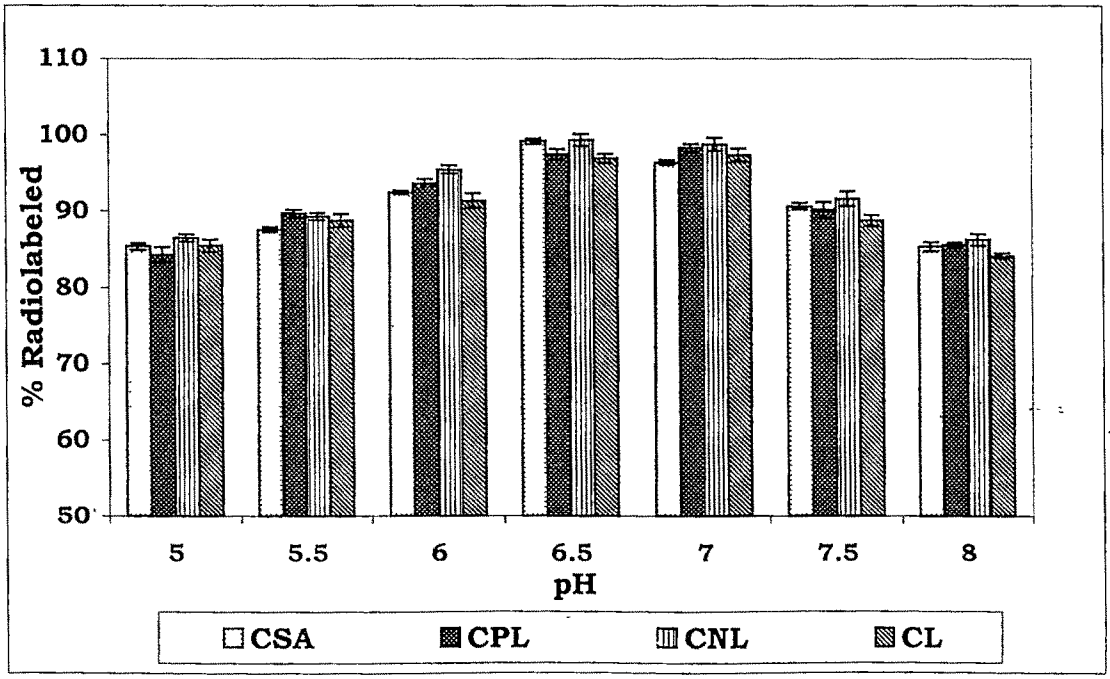


Table 10.8 Effect of incubation time on radiolabeling efficiency of leuprolide acetate and its liposomal formulations

pH	Percent Radiolabeled (± S.E.M)			
	Leuprolide	LL	SLL5000	SLL2000
0	90.3±1.3	90.2±2.2	90.6±2.1	91.7±1.5
5	96.9±1.6	98.9±1.2	98.9±1.6	98.3±1.3
15	96.2±2.0.	98.6±1.8	98.5±1.5	98.7±1.6
20	96.9±1.3	99.1±1.7	98.2±1.6	98.2±1.2
30	97.0±1.5	99.1±1.0	99.1±1.8	98.6±2.0
40	96.9±1.9	98.2±2.1	99.1±2.0	98.5±1.8

Figure 10.5 Effect of incubation time on radiolabeling efficiency of leuprolide and its liposomal formulations

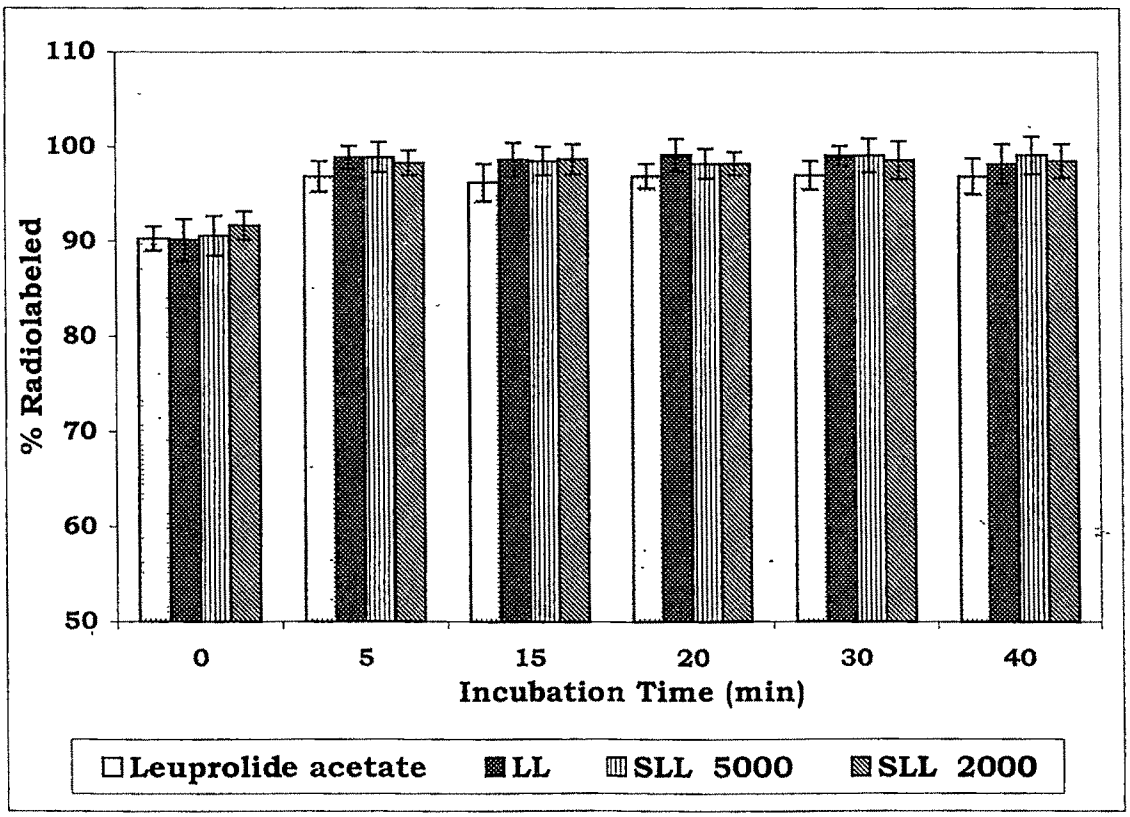


Table 10.9 Effect of $SnCl_2.2H_2O$ concentration on radiolabeling efficiency of leuprolide and its liposomal formulations

$Sncl_2.$ $2H_2O$ µg/ml	leuprolide			LL			SLL5000			SLL2000		
	% Bound	% colloids	% free	% Bound	% colloids	% free	% Bound	% colloids	% free	% Bound	% colloids	% free
25	70.23	1.26	28.51	75.62	1.02	23.36	78.86	0.95	20.19	80.23	0.85	18.92
50	86.68	2.89	10.43	86.53	0.98	12.49	80.26	1.02	18.72	82.68	0.96	16.36
100	96.23	1.03	2.74	98.6	1.32	1.06	97.86	1.23	0.91	98.02	1.23	1.75
150	92.93	2.70	6.37	91.9	1.21	6.89	92.9	3.62	3.48	91.3	1.76	6.94
200	93.62	6.2	0.18	95.02	4.30	0.68	92.23	4.02	3.75	92.68	4.59	2.73
300	90.36	8.69	0.95	92.88	6.81	0.31	91.02	6.26	2.72	92.23	7.02	0.75

Table 10.10 Stability studies of radiolabeled leuprolide and its liposomal formulations in saline

	% Radiolabeling Efficiency						
	15 min	**30 min**	**1 h**	**2 h**	**4 h**	**6 h**	**24 h**
Leuprolide	96.2	96.9	98.7	96.7	94.4	94.2	92.3
LL	98.6	99.1	99.1	98.4	99.2	98.2	98.6
SLL5000	98.5	99.1	99.1	97.8	99.1	99.2	98.5
SLL2000	98.7	99.2	98.6	97.2	97.0	97.23	96.3

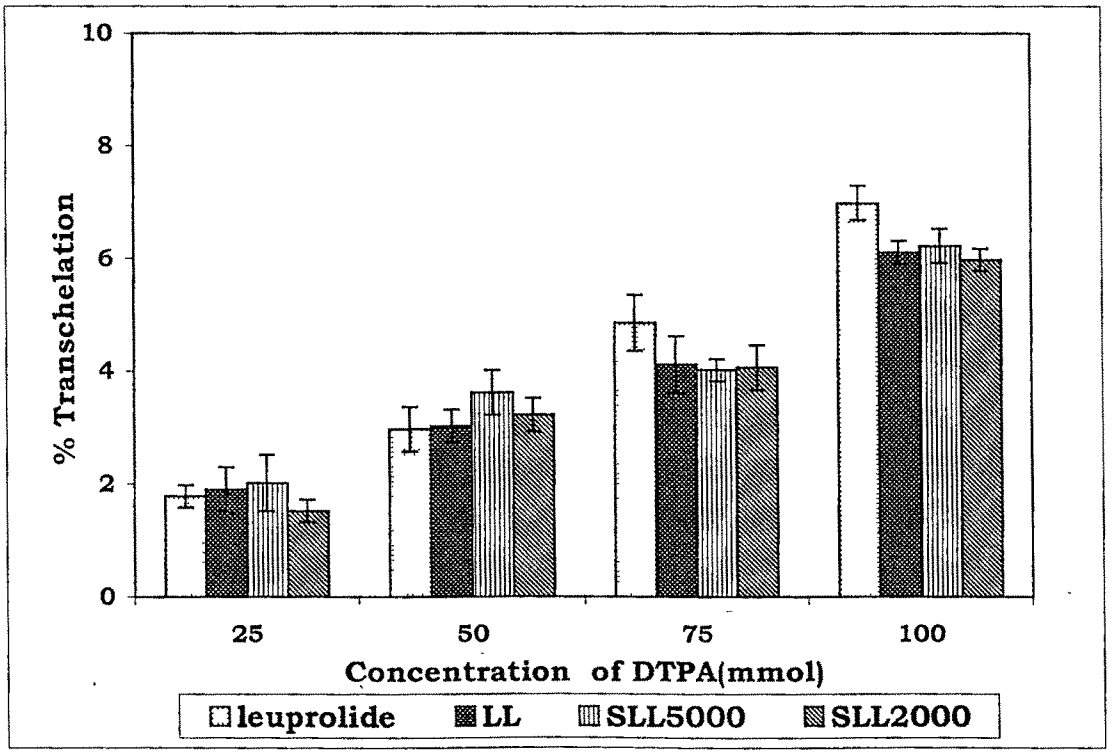
Table 10.11 Stability studies of radiolabeled leuprolide and its liposomal formulations in serum

	% Radiolabeling Efficiency						
	15 min	**30 min**	**1 h**	**2 h**	**4 h**	**6 h**	**24 h**
Leuprolide	96.9	96.8	96.7	96.0	95.8	95.9	96.0
LL	98.3	98.0	98.1	97.6	97.9	97.6	97.8
SLL5000	98.7	98.3	98.1	97.2	97.1	97.3	97.1
SLL2000	98.0	98.2	98.0	97.6	97.8	97.6	97.9

Table 10.12 DTPA challenging test of leuprolide and its liposomal formulations

Conc. of DTPA (mM)	% Transchelation			
	Leuprolide	LL	SLL5000	SLL2000
25	1.78±0.2	1.9±0.4	2.02±0.5	1.52±0.2
50	2.97±0.4	3.02±0.3	3.62±0.4	3.23±0.3
75	4.86±0.5	4.12±0.5	4.02±0.2	4.06±0.4
100	6.99±0.3	6.12±0.2	6.23±0.3	5.98±0.2

Figure 10.6 DTPA challenging test of leuprolide and its liposomal formulations



10.7.6 OPTIMIZATION OF RADIOLABELING OF DNA AND ITS LIPOSOMAL FORMULATIONS

The radiolabeling of DNA and its liposomal formulations (DL, SDL5000, SDL2000, and CSDL5000) were done with Technetium-99m (^{99m}Tc) by simple reduction method as described earlier (Richardson et. al., 1977). The pertechnetate used for the study was first reduced to its lower valency state using stannous chloride dihydrate and then pH was adjusted to neutral before mixing with the DNA/liposome suspension. The radiolabeling was optimised by taking three factors into account. i.e. pH of the complex, incubation time and stannous chloride dihydrate concentration. The pH of the labelled complex was increased from 5 to 8 and its effect on labelling efficiency was studied. The radiolabeled complexes were incubated for various time periods and the effect of incubation time on labeling efficiency was determined keeping other variables constant. The effect of $SnCl_2.2H_2O$ concentration on the labeling efficiency was also studied to obtain the optimum concentration needed for maximum labeling.

1ml of ^{99m}Tc pertechnetate (2mCi/ml) was mixed with specific amount of stannous chloride solution (1mg/ml) and the pH was adjusted suitably using sodium bicarbonate solution. To this mixture, 1ml of DNA solution (200µg/ml) or 1ml of liposome suspension (50-60mgm of lipid) containing 200µg of DNA was added and incubated for specific time period at room temperature. The radiochemical purity of labeled complex was estimated by ascending instant thin layer chromatography using 100% acetone or 0.9% sodium chloride as developing solvents.

Table 10.13 Effect of pH on radiolabeling efficiency of DNA and its liposomal formulations

pH	Percent Radiolabeled (± S.E.M)				
	DNA	DL	SDL5000	SDL2000-CC-PE	CSDL2000-CC-PE
5.0	80.23±1.42	80.23±4.98	81.62±4.23	80.68±2.65	81.63±2. 33
5.5	89.56±3.63	85.68±5.69	84.23±3.69	83.68±3.26	85.69±3.69
6.0	90.23±2.98	90.23±3.95	90.68±3.58	89.23±1.23	87.95±2.99
6.5	95.36±3.02	96.99±4.02	96.36±2.96	96.58±3.45	95.89±1.98
7.0	96.78±1.99	97.02±1.89	97.66±3.02	97.66±2.05	97.02±3.87
7.5	89.62±2.89	90.63±2.99	91.65±3.69	90.69±3.62	90.12±3.48
8.0	80.36±3.98	83.69±4.02	87.96±4.56	88.63±4.08	87.69±2.93

Figure 10.7 Effect of pH on radiolabeling efficiency of DNA and its liposomal formulations

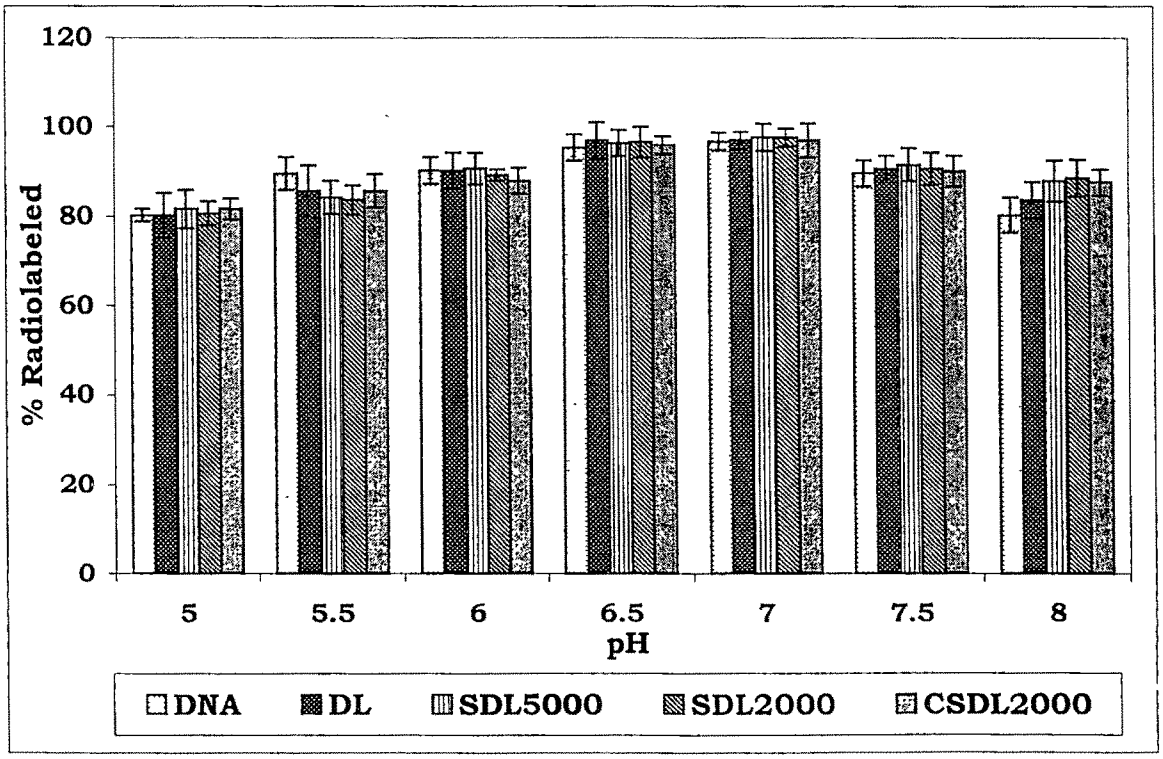


Table 10.14 Effect of incubation time on radiolabeling efficiency of DNA and its liposomal formulations

pH	Percent Radiolabeled (± S.E.M)				
	DNA	DL	SDL5000	SDL2000	CSDL2000
0	91.23±1.11	90.23±1.36	92.13±1.22	92.68±0.89	90.58±0.99
5	92.36±0.94	94.7±1.78	98.9±0.98	98.9±1.56	97.62±1.23
15	94.23±0.85	97.8±0.63	98.7±1.46	98.5±1.23	98.63±0.97
20	97.68±1.02	97.6±0.99	98.5±0.65	98.3±1.26	98.02±1.36
30	95.63±1.06	97.8±1.02	97.5±1.23	98.5±0.99	98.23±2.02
40	95.32±0.77	97.23±0.65	97.6±0.68	98.62±1.32	98.25±1.98

Table 10.8 Effect of incubation time on radiolabeling efficiency of DNA and its liposomal formulations

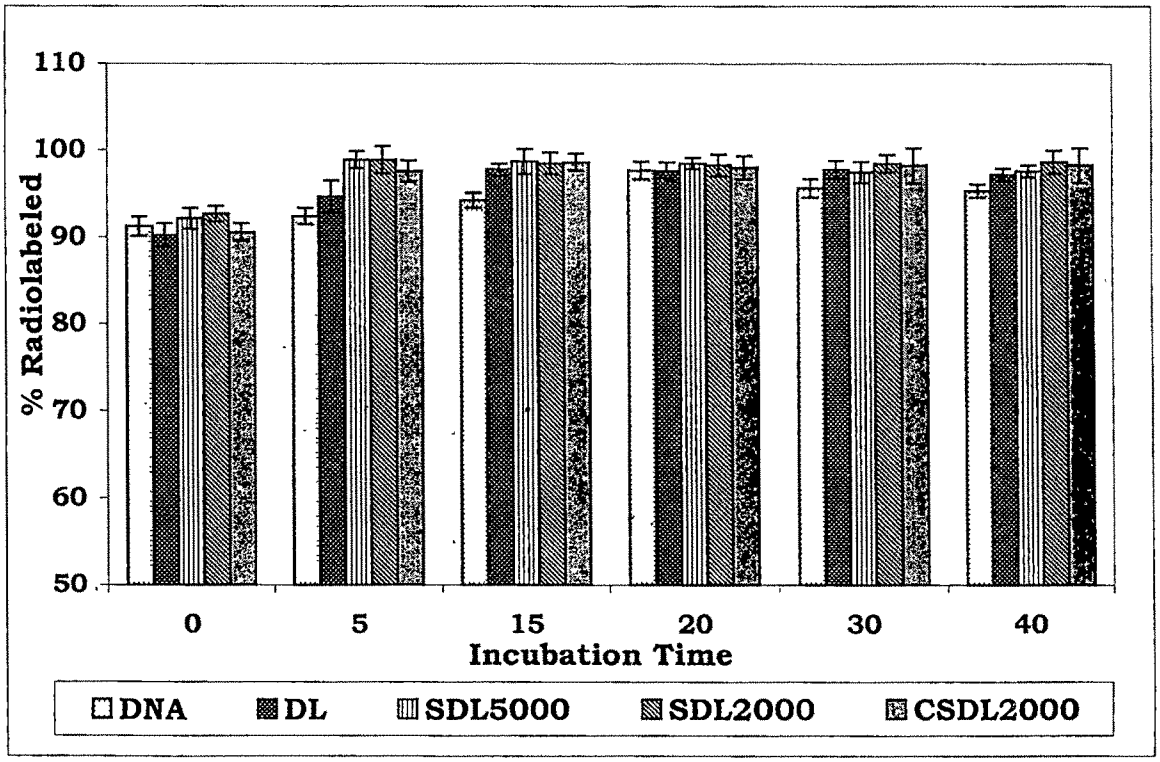


Table 10.15 Effect of $SnCl_2.2H_2O$ concentration on radiolabeling efficiency of DNA and its liposomal formulations

$Sncl_2$ $2H_2O$ (μg)	DNA			DL			SDL5000			SDL2000			CSDL2000		
	% Bound	% Colloid	% Free	% Bound	% Colloid	% Free	% Bound	% Colloid	% Free	% Bound	% Colloid	% Free	% Bound	% Colloid	% Free
25	72.56	1.03	26.41	78.62	0.89	20.49	79.23	0.65	20.12	74.23	0.21	25.56	78.92	0.36	20.72
50	85.98	1.67	11.35	82.62	1.32	16.06	85.68	1.10	13.22	84.23	0.45	15.32	85.23	0.92	13.85
100	98.6	1.02	0.38	97.8	0.68	1.52	98.9	0.65	0.45	98.9	0.65	0.45	97.62	1.23	1.15
150	94.23	4.69	1.08	97.23	2.30	0.47	96.62	2.87	0.51	96.82	3.02	0.16	95.23	4.65	0.12
200	94.12	4.98	0.9	92.58	6.20	4.85	93.92	5.24	0.84	92.89	6.25	0.86	92.02	6.58	1.4
300	92.98	6.98	0.04	91.84	7.98	0.18	92.23	7.58	0.19	92.56	7.26	0.18	92.89	7.01	0.1

Table 10.16 Stability studies of radiolabeled DNA and its liposomal formulations in saline

	% Radiolabeling Efficiency						
	15 min	**30 min**	**1 h**	**2 h**	**4 h**	**6 h**	**24 h**
DNA	94.23	95.63	95.23	95.63	95.42	94.23	90.68
DL	97.8	97.8	96.0	97.8	96.0	94.9	94.6
SDL5000	98.7	97.5	98.5	97.5	97.1	97.62	97.02
SDL2000	98.5	98.5	98.3	98.8	97.0	97.23	96.8
CSDL2000	98.63	98.23	98.6	97.2	96.6	96.86	94.66

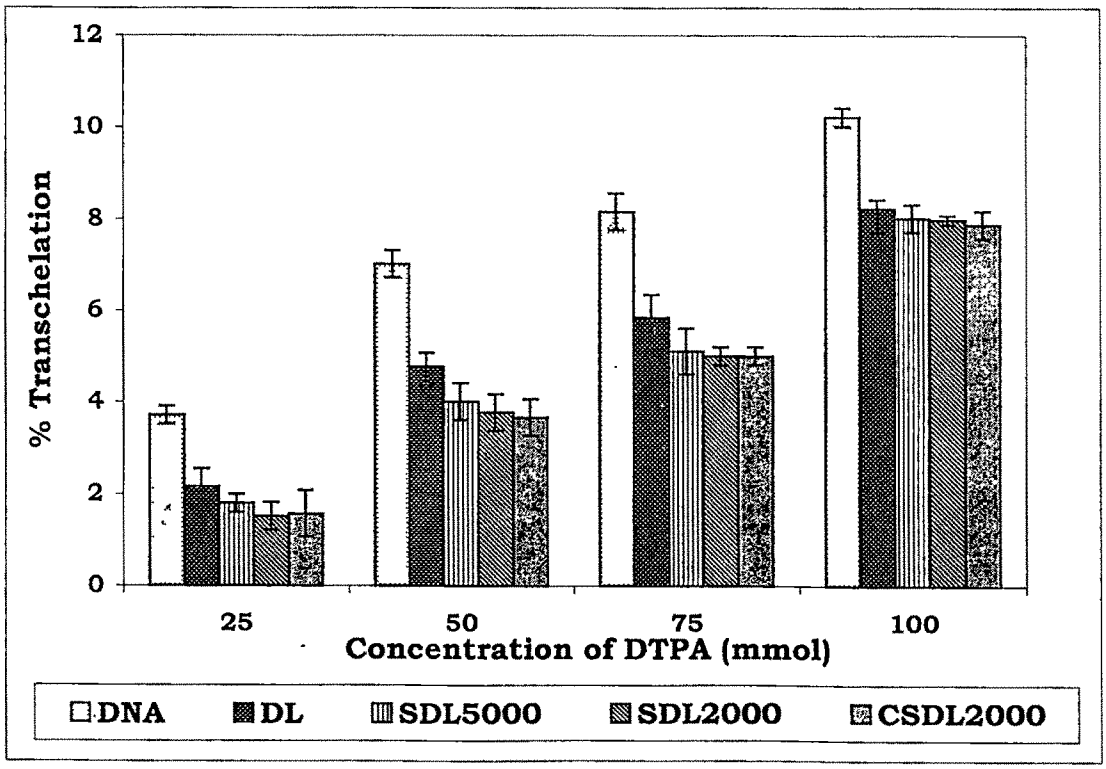
Table 10.17 Stability studies of radiolabeled DNA and its liposomal formulations in serum

	% Radiolabeling Efficiency						
	15 min	**30 min**	**1 h**	**2 h**	**4 h**	**6 h**	**24 h**
DNA	94.23	95.63	95.02	94.23	93.26	93.02	91.23
DL	97.8	97.8	96.53	96.49	96.23	96.23	96.08
SDL5000	98.7	97.5	98.27	97.89	97.63	97.02	97.09
SDL2000	98.5	98.5	98.67	98.63	97.02	98.23	97.18
CSDL2000	98.63	98.23	98.29	98.02	97.63	97.59	97.39

Table 10.18 DTPA challenging test of DNA and its liposomal formulations

Conc. Of DTPA (mM)	% Transchelation				
	DNA	DL	SDL5000	SDL2000	CSDL2000
25	3.72±0.2	2.15±0.4	1.8±0.2	1.52±0.3	1.58±0.5
50	7.02±0.3	4.78±0.3	4.02±0.4	3.78±0.4	3.67±0.4
75	8.16±0.4	5.86±0.5	5.12±0.5	5.02±0.2	5.02±0.2
100	10.23±0.2	8.23±0.2	8.02±0.3	7.98±0.1	7.88±0.3

Figure 10.9 DTPA challenging test of DNA and its liposomal formulations



10.8 RESULTS AND DISCUSSION

10.8.1 RADIOLABELING OF CYCLOSPORINE AND ITS LIPOSOMES

The cyclosporine and its liposomes were labeled with high efficiency by the direct labeling technique using reduced ^{99m}Tc. Data on radiochemical purity and stability of the labeled complex as obtained by ascending chromatography are detected on the chromatograms by using 0.9% saline or 100 % acetone. The radiochemical impurities are free $^{99m}TcO_4^-$ and hydrolyzed ^{99m}Tc (radiocolloids) in the ^{99m}Tc-labeled complexes. The pertechnetate used for the study was first reduced to its lower valency state using stannous chloride dihydrate and then pH was adjusted to neutral before mixing with the cyclosporine/liposome suspension. The radiolabeling was optimized by taking three factors into account. i.e. pH of the complex, stannous chloride dihydrate concentration and incubation time.

10.8.1.1 pH of the complex

The figure 10.1 depicts the effect of pH on labeling efficiency. As the pH increases from five to seven, the radiolabeling also increases from 85.35 % to 99.17 % for cyclosporine and 84 % to 98 % for the liposomes. Further increase in the pH led to reduction in the labeling efficiency. The maximum labeling achieved for cyclosporine/liposomes in between the pH 6.5 to 7.0.

10.8.1.2 Incubation time

To find out the relationship between the incubation time and radiolabeling efficiency, the cyclosporine/ liposomes were mixed with the reduced ^{99m}Tc and incubated at various time intervals. The labeling efficiency was calculated after each time point (table 10.2). The figure 10.2 shows the effect of incubation time on labeling efficiency. The incubation time required for maximum labeling efficiency was found to be 15min for cyclosporine and its liposomes. Further increase in incubation time does not increase the labeling efficiency considerably.

10.8.1.3 $SnCl_2.2H_2O$ concentration

By varying the amount of $Sncl_2.2H_2O$ from 25 to 400µg, but keeping the amount of drug and liposome to be labeled as constant at pH 6.5-7.0, the influence of labeling yield was shown in table 10.3. The labeling yield was

only 8285 % for all the formulations and drug when 25µg of the $Sncl_2.2H_2O$ was added; it was 97-99 % when 100µg of $Sncl_2.2H_2O$ was added. Further increase in the amount of stannous chloride led to the reduction in the yield. It is interesting to note that at the lowest concentration of $Sncl_2.2H_2O$, the remaining activity was as free $^{99m}TcO_4^-$, whereas with the concentration above 200 µg, it was as radio colloids, which were shown in table 10.3.

10.8.1.4 Stability of the labeled complex

Stability of the labeled complex with time was studied in saline and in serum (rabbit) at 37°C as shown in table 10.4 and table 10.5. Even after a period of 24 h incubation the presence of > 94 % labeled compound and only 5-6 % decrease of the labeled product signifies not only the high stability of the radio labeled product but also its suitability for in vivo use. High binding affinity of the ^{99m}Tc-labeled cyclosporine and its liposomes was ascertained by incubating the tagged compound with DTPA at different molar ratios as shown in table 10.6 and figure 10.3. DTPA at a molar concentration of 25 mmol decreased labeling by only 1-2 %. However, when DTPA was increased to 100mmol, the labeling % was reduced by about 6-8 %. The observation could be appreciated due to higher strength and binding affinity of ^{99m}Tc with cyclosporine and its liposomal formulations.

10.8.2 RADIOLABELING OF LEUPROLIDE AND ITS LIPOSOMES

The leuprolide and its liposomes were labeled with high efficiency by the direct labeling technique using reduced ^{99m}Tc. Data on radiochemical purity and stability of the labeled complex as obtained by ascending chromatography are detected on the chromatograms by using 0.9% saline or 100 % acetone. The radiochemical impurities are free$^{99m}TcO_4^-$ and hydrolyzed ^{99m}Tc (radiocolloids) in the ^{99m}Tc-labeled complexes. The pertechnetate used for the study was first reduced to its lower valency state using stannous chloride dihydrate and then pH was adjusted to neutral before mixing with the leuprolide acetate/liposome suspension. The radiolabeling was optimized by taking three factors into account. i.e., pH of the complex, stannous chloride dihydrate concentration and incubation time.

10.8.2.1 pH of the complex

The table 10.7 and figure 10.4 depicts the effect of pH on labeling efficiency. As the pH increases from five to seven, the radiolabeling also increases from 70.56 % to 96.93 % for leuprolide and 74 % to 98 % for the liposomes. Further increase in the pH led to reduction in the labeling efficiency. The maximum labeling achieved for leuprolide/liposomes in between the pH 6.5 to 7.0.

10.8.2.2 Incubation time

To find out the relationship between the incubation time and radiolabeling efficiency, the leuprolide acetate/liposomes were mixed with the reduced ^{99m}Tc and incubated at various time intervals. The labeling efficiency was calculated after each time point (table 10.8). The figure 10.5 shows the effect of incubation time on labeling efficiency. The incubation time required for maximum labeling efficiency was found to be 15min for leuprolide and its liposomes. Further increase in incubation time does not increase the labeling efficiency considerably.

10.8.2.3 $SnCl_2.2H_2O$ concentration

By varying the amount of $Sncl_2.2H_2O$ from 25 to 300µg, but keeping the amount of drug and liposome to be labeled as constant at pH 6.5-7.0, the influence of labeling yield was shown in table 10.9. The labeling yield was only 70-80 % for all the formulations and drug when 25µg of the $Sncl_2.2H_2O$ was added; it was 96-98 % when 100µg of $Sncl_2.2H_2O$ was added. Further increase in the amount of stannous chloride led to the reduction in the yield. It is interesting to note that at the lowest concentration of $Sncl_2.2H_2O$, the remaining activity was as free $^{99m}TcO_4^-$, whereas with the concentration above 200 µg, it was as radio colloids, which were shown in table 10.9.

10.8.2.4 Stability of the labeled complex

Stability of the labeled complex with time was studied in saline and in serum (rabbit) at 37°C as shown in table 10.10 and table 10.11. Even after a period of 24 h incubation the presence of > 94 % labeled compound and only 5-6 % decrease of the labeled product signifies not only the high stability of the radio labeled product but also its suitability for in vivo use. High binding affinity of the ^{99m}Tc-labeled leuprolide acetate and its

liposomes was ascertained by incubating the tagged compound with DTPA at different molar ratios as shown in table 10.12 and figure 10.6. The percentage transchelation was found to be as little as 1% to 2% at 25mM concentration of DTPA, and even at high concentration of 100mM, the maximum percentage transchelation was found to be only 6% to 7%. The observation could be appreciated because of higher strength and binding affinity of ^{99m}Tc with leuprolide acetate and its liposomal formulations.

10.8.3 RADIOLABELING OF DNA AND ITS LIPOSOMES

The DNA and its liposomes were labeled with high efficiency by the direct labeling technique using reduced ^{99m}Tc. Data on radiochemical purity and stability of the labeled complex as obtained by ascending chromatography are detected on the chromatograms by using 0.9% saline or 100 % acetone. The radiochemical impurities are free $^{99m}TcO_4^-$ and hydrolyzed ^{99m}Tc (radiocolloids) in the ^{99m}Tc-labeled complexes. The pertechnetate used for the study was first reduced to its lower valency state using stannous chloride dihydrate and then pH was adjusted to neutral before mixing with the cyclosporine/liposome suspension. The radiolabeling was optimized by taking three factors into account. i.e. pH of the complex, stannous chloride dihydrate concentration and incubation time.

10.8.3.1 pH of the complex

The figure 10.7 and table 10.13 depicts the effect of pH on labeling efficiency. As the pH increases from five to seven, the radiolabeling also increases from 80.23 % to 96.78 % for DNA and 80 % to 97 % for the liposomes. Further increase in the pH led to reduction in the labeling efficiency. The maximum labeling achieved for DNA/liposomes in between the pH 6.5 to 7.0.

10.8.3.2 Incubation time

To find out the relationship between the incubation time and radiolabeling efficiency, the DNA/ liposomes were mixed with the reduced ^{99m}Tc and incubated at various time intervals. The labeling efficiency was calculated after each time point (table 10.14). The figure 10.8 shows the effect of incubation time on labeling efficiency. The incubation time required for maximum labeling efficiency was found to be 20min for DNA, 15 minutes for conventional liposomes and 5 minutes for sterically stabilized liposomes.

Further increase in incubation time does not increase the labeling efficiency considerably.

10.8.3.3 $SnCl_2.2H_2O$ concentration

By varying the amount of $Sncl_2.2H_2O$ from 25 to 300µg, but keeping the amount of drug and liposome to be labeled as constant at pH 6.5-7.0, the influence of labeling yield was shown in table 10.15. The labeling yield was only 70-80 % for all the formulations and drug when 25µg of the $Sncl_2.2H_2O$ was added; it was 96-98 % when 100µg of $Sncl_2.2H_2O$ was added. Further increase in the amount of stannous chloride led to the reduction in the yield. It is interesting to note that at the lowest concentration of $Sncl_2.2H_2O$, the remaining activity was as free $^{99m}TcO_4^-$, whereas with the concentration above 200 µg, it was as radio colloids, which were shown in table 10.15.

10.8.3.4 Stability of the labeled complex

Stability of the labeled complex with time was studied in saline and in serum (rabbit) at 37°C as shown in table 10.16 and table 10.17. Even after a period of 24 h incubation the presence of > 90 % labeled compound and only 3-10 % decrease of the labeled product signifies not only the high stability of the radio labeled product but also its suitability for in vivo use. High binding affinity of the ^{99m}Tc-labeled DNA and its liposomes was ascertained by incubating the labeled compound with DTPA at different molar ratios as shown in table 10.18 and figure 10.9. DTPA at a molar concentration of 25 mmol decreased labeling by only 1-3 %. However, when DTPA was increased to 100mmol, the labeling % was reduced by about 7-10 %. The observation could be appreciated due to higher strength and binding affinity of ^{99m}Tc with DNA and its liposomal formulations.

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