Chapter 7

Radiolabeling of Liposomes

7. Radiolabeling of liposomes

7.1 Introduction

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 with low toxicity (Saha, 1993).

In practice, the majority of radio pharmaceuticals are used for diagnosis (Mishra et. al. 1999), but there are number of radionuclides available for the treatment of some disorders, especially cancer (Babbar and Sharma, 2003). In the typical radiopharmaceutical formulation, the quantities of radionuclides and pharmaceutical agents used are normally quite less. The radiopharmaceutical differs from conventional pharmaceutical in that it is not intended to elicit a pharmacological response due to the sub therapeutic doses administered. Hence, the radiopharmaceutical does not disturb the normal physiological process being measured, functions as a true tracer, and they are generally free from hypersensitivity reactions.

The emergence of scintigraphy or imaging techniques for studying the Biodistribution patterns in the sixties and seventies has lead to the increase in the popularity of the application of nuclear medicine. These techniques allow non invasive Biodistribution study by usage of an external detection system viz. Gamma camera (Single Photon Emission computed Tomography – SPECT). SPECT imaging represents methods for acquiring and processing the scintigraphic data to reconstruct a three dimensional tomographic image displaying a distribution of radioactivity within certain organ system using emitted gamma rays upon administration of a radio tracer (Sorenson and Phelps, 1987: Budinger, 1980). Gamma imaging has lead to an increase in the demand for short lived radiotracers which can be safely administered in larger doses with minimal radiation side effects. For biological experiments, the radionuclides are linked to the compounds of interest by various techniques. The effective binding of radionuclide to the compound is determined by the quality control tests such as labeling efficiency, stability

of radiolabeled complexes, challenge tests using substances having high affinity to the radionuclides and serum stability.

Nearly 80 % of all radiopharmaceuticals used in nuclear medicine are ^{99m} Tc-labeled compounds. Technetium is prepared by the following reaction from Uranium (^{235} U).

$$^{235}U \longrightarrow {}^{99}Mo \longrightarrow {}^{99m}Tc$$

The common methods of separation of ^{99m}Tc and ^{99}Mo

1. Column chromatography over acidic alumina
2. Solvent extraction of ^{99m}Tc with methyl ethyl ketone.
3. Sublimation of Tc oxides from Mo compounds

The reason for such a predominant position of ^{99m} Tc in clinical use is its extremely favorable physical and radiation characteristics. The 6 h physical half life and the little amount of electron emission permits 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 principle involved in the measurement of radioactivity is as follows. The gamma rays emitted by the isotopes enter a stainless steel casing and generate electrons which are absorbed by the sodium iodide (NaI) crystals. The NaI crystal undergoes excitation and further de-excitation to produce a flash of light. This flash of light passes through an optically coupled photomultiplier tube. In the multiplier tube, the intensity of light is enhanced and passes through a pre-amplifier and linear amplifier and consequently to the pulse height analyzer. The signal are then tuned in a tuner and recorded in a recorder in case of gamma camera. The gamma camera is equipped with a scaler instead of recorder. In scaler, the signals are converted into digits in terms of counts.

Quality control is an important aspect in the formulation and use of radiopharmaceuticals as it decides the efficacy for the purpose used. Before using the radionuclide for linking to the compound, the quality control testing is necessary to assure the efficacy of radionuclide. They include – radioactivity, radionuclide concentration, radionuclide purity and identity, radiochemical purity, chemical purity, sterility, apyrogenicity, absence of foreign particulate matter, particle size (Babbar and Sharma, 2003).

Chemistry of Technetium

Technetium is a transition metal of silvery grey color belonging to group VIIIB (Mn, Tc and Re) and has the atomic number 43. No stable isotope of technetium exists in nature. The ground state ^{99m}Tc has a half-life of 2.1 x 10^5 years. The electronic structure of the neutral technetium atom is $1s^2 2s^2 2p^6 3s^2 3p^6 3d^{10} 4s^2 4p^6 4d^6 5s^1$. Technetium can exist in 8 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 e.g., Tc^{1+}, complexed with six isonitrile groups in ^{99m}Tc-sestamibi. Otherwise they are oxidized to 4+ state and finally to the 7+ state (Saha, 1993).

Reduction of $^{99m}TcO_4^-$

The chemical form of ^{99m}Tc available from the Molybdenum generator is sodium pertechnetates (^{99m}Tc-$NaTcO_4$). The pertechnetates ion $^{99m}TcO_4^-$, having the oxidation state 7+ for ^{99m}Tc, resembles the permanganate ion MnO_4^-, 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 agent in acidic medium in most preparations of ^{99m}Tc-labeled compounds.

Labeling with reduced Technetium.

The reduced technetium species are chemically reactive and combine with a wide variety of compounds, which usually donates a 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.

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 hydrolyzed 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.

7.2 *Materials*

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

7.3 Radiolabeling of liposomal formulations

The Radiolabeling of Paclitaxel (PCL) and Irinotecan Hydrochloride (IH) and their liposomal formulations with reduced ^{99m}Tc were carried out as per the procedure given below.

Drug and their liposomal formulations were radiolabeled with ^{99m}Tc by reduction with stannous chloride similar to the methods reported earlier (Richardson et. al. 1977; Theobald, 1990). Briefly, the optimized quantity of stannous chloride was added to the respective drug solution or liposomal formulation. Then the pH was adjusted if required to around 6.5-7.0 using 0.5 M sodium bicarbonate. Then ^{99m}Tc (2 mCi) was added to the above system and incubated at room temperature for optimized time periods (20 min).

Labeling efficiency

To study the labeling efficiency of ^{99m}Tc with the drugs/formulations, the ^{99m}Tc labeled complex was subjected to ascending instant thin layer chromatography using ITLC-SG strips 1x12 cm (made of glass fiber impregnated with silica gel, Gelman sciences, Inc., Ann Arbor, MI) as a stationary phase and 100% acetone or 0.9% saline as mobile phase. Approximately 2 µl of the radiolabeled complex was applied at a point 1cm from one end of an ITLC-SG strip. The strip was developed in 100% acetone or 0.9 % saline and the solvent front was allowed to reach 8 cm from the origin. The strip was cut horizontally into 2 halves and the radio activity in each segment was determined in a well type gamma ray counter (Type: CAPRAC-R, Capintec, Inc., USA). The free pertechnetate that moved with the solvent (R_f =0.9) was determined. The reduced / hydrolyzed (R/H) technetium along with the labeled complex remained at the point of application. The amount of reduced/hydrolyzed technetium 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 where both the 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-labeled complex was calculated.

Stability study of ^{99m} Tc labeled complex

The *in-vivo* stability of radiolabeled complex was tested in serum and physiological saline (Chauhan et. al., 1993). The study was accomplished by incubating an aliquot of 0.1ml of labeled complex and 0.9ml of serum or saline at 37°C for 24 h. small aliquots were withdrawn during incubation and subjected to ITLC using 100% acetone as the mobile phase. Strips were dried and cut into 2 pieces and radio activity in both the pieces was counted. Any increase in pertechnetate percentage was considered as the degree of degradation of the labeled complex (Gulati et. al., 2005; Reddy et. al., 2004).

DTPA and Cysteine challenge test

The binding affinity of the labeled complexes was confirmed by the transchelation using DTPA and Cysteine. The stability of the complexes was examined by challenging with DTPA and Cysteine at different concentrations (Mishra et. al., 2002). The DTPA and Cysteine challenge assays involved incubation of the labeled complex with different concentrations of transchelators (25 -100mM) at room temperature for a period of 1 h respectively. The effect of DTPA and Cysteine 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 (R_f = 0.9 -1.0) and all know chemical forms of ^{99m} Tc-DTPA and ^{99m} Tc-cysteine complexes (R_f = 0.7-1.0) from the ^{99m} Tc-liposome complex which remained at the point of application (R_f = 0). After developing, each paper was cut into 2 halves and each half was counted for radioactivity gamma ray counter.

7.4 Optimization of Radiolabeling of PCL and its liposomal formulations

PCL and its liposomal formulations (conventional liposomes and pH sensitive liposomes) were labeled with ^{99m} Tc by simple reduction method (Richardson et. al., 1977). The pertechnetate used for the study was first reduced to its lower valence state using stannous chloride dehydrate and then pH was adjusted to neutral before mixing with the PCL / liposomal suspension. The Radiolabeling was optimized by taking 3 factors into account i. e. pH of the complex, incubation time and stannous chloride dehydrate 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 stannous chloride concentration on the labeling efficiency was also studied to obtain the optimum concentration needed for maximum labeling. The Radiolabeling procedure for PCL and its liposomal formulations is given below.

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

Table 7.1. Effect of pH on radiolabeling efficiency of PCL and its liposomal formulations

pH	Percent radiolabeled (±S.E)		
	PCL	CLPT	PSPT
5.0	70.34 ± 2.65	71.45 ± 3.01	NA
5.5	76.67 ± 2.76	79.96 ± 2.95	NA
6.0	89.12 ± 2.11	90.34 ± 2.56	90.77 ± 1.86
6.5	94.23 ± 1.84	95.00 ± 2.04	95.09 ± 1.68
7.0	97.96 ± 1.62	98.38 ± 1.59	98.64 ± 1.03
7.5	97.15 ± 2.04	97.33 ± 1.88	98.01 ± 1.33
8.0	96.87 ± 2.36	96.98 ± 2.05	97.23 ± 1.89

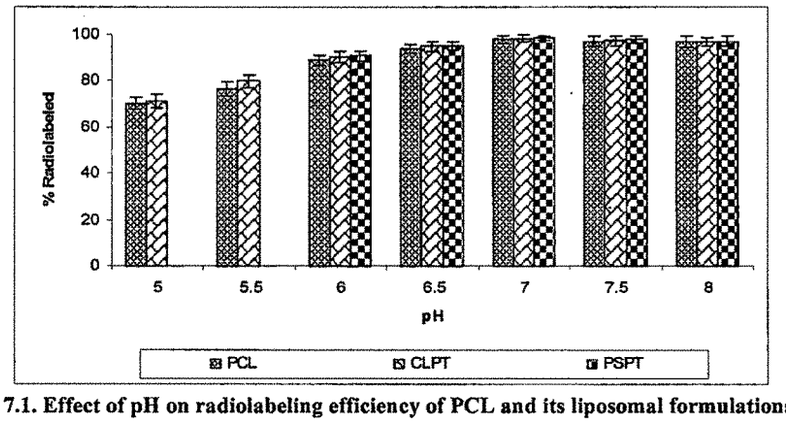

Figure 7.1. Effect of pH on radiolabeling efficiency of PCL and its liposomal formulations

Table 7.2. Effect of incubation time on radiolabeling efficiency of PCL and its liposomal formulations

Incubation time (min)	Percent radiolabeled (±S.E)		
	PCL	CLPT	PSPT
0	88.65 ± 2.02	89.13 ± 2.10	89.66 ± 1.90
5	90.43 ± 1.73	90.91 ± 1.85	93.41 ± 1.75
10	94.29 ± 1.61	96.14 ± 1.54	96.76 ± 1.51
20	95.11 ± 1.44	97.07 ± 0.94	98.37 ± 0.76
30	97.68 ± 1.42	98.34 ± 1.29	98.54 ± 1.11
45	98.45 ± 0.94	98.52 ± 0.78	98.87 ± 0.75
60	98.51 ± 1.36	98.68 ± 1.03	98.94 ± 0.86

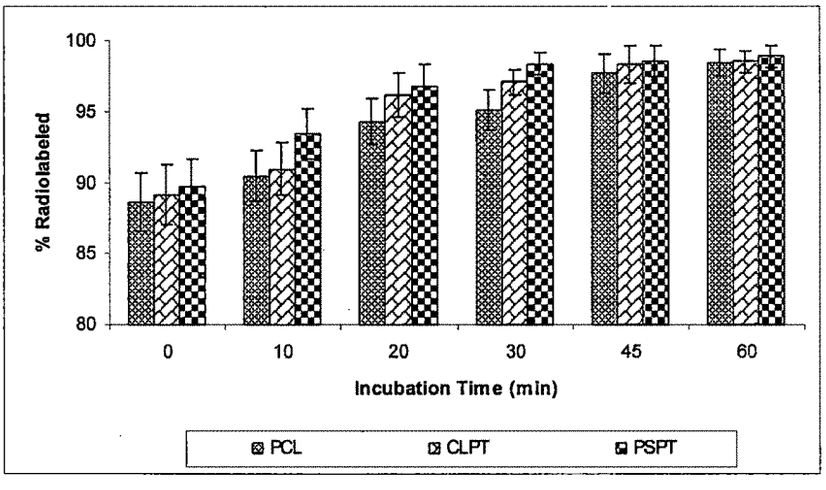

Figure 7.2. Effect of incubation time on radiolabeling efficiency of PCL and its liposomal Formulations.

Table 7.3. Effect of $SnCl_2.2H_2O$ on radiolabeling efficiency of PCL and its liposomal Formulations.

Conc. Of $SnCl_2.2H_2O$ (µg)	PCL			CLPT			PSPT		
	% Bound	% Colloid	% Free	% Bound	% Colloid	% Free	% Bound	% Colloid	% Free
25	84.57	1.22	14.21	85.23	0.98	13.79	85.76	0.95	13.29
50	89.12	1.36	9.52	89.72	1.33	8.95	90.52	1.26	8.22
75	92.33	1.58	6.09	93.10	1.42	5.48	93.98	1.40	4.62
100	97.98	1.69	0.33	98.09	1.54	0.37	98.84	0.94	0.22
150	94.76	4.47	0.77	94.81	4.16	1.03	91.81	5.56	2.63
200	90.68	7.36	1.96	90.39	8.17	1.44	89.73	9.17	1.10

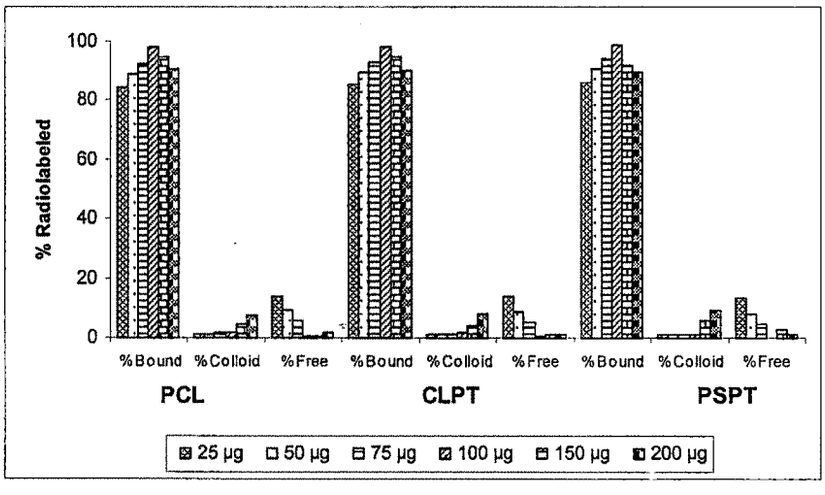

Figure 7.3. Effect of $SnCl_2.2H_2O$ on radiolabeling efficiency of PCL and its liposomal formulations.

Table 7.4. Stability study of radiolabeled PCL and its liposomal formulations in saline.

Formulations	% Radiolabeling Efficiency (± S.E.)						
	0 min	30 min	1 h	2 h	4 h	6 h	24 h
PCL	97.39 ± 0.48	98.12 ± 0.56	98.68 ± 0.33	97.83 ± 0.65	97.43 ± 0.58	97.56 ± 0.94	95.12 ± 0.78
CLPT	97.13 ± 0.66	98.46 ± 0.54	98.71 ± 0.46	97.98 ± 0.28	97.86 ± 0.49	97.20 ± 0.0.65	96.51 ± 0.72
PSPT	97.86 ± 0.44	98.48 ± 0.68	98.85 ± 0.18	98.31 ± 0.38	97.99 ± 0.71	97.66 ± 0.76	96.84 ± 0.52

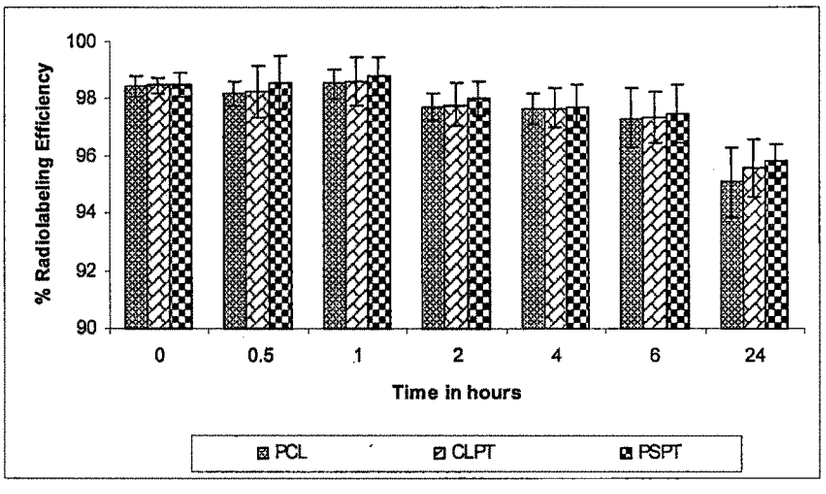

Figure 7.4. Stability study of radiolabeled PCL and its liposomal formulations in saline.

Table 7.5. Stability study of radiolabeled PCL and its liposomal formulations in serum

Formulations	% Radiolabeling Efficiency (± S.E.)						
	0 min	30 min	1 h	2 h	4 h	6 h	24 h
PCL	98.12 ± 0.36	98.43 ± 0.67	98.48 ± 0.42	97.12 ± 0.18	97.04 ± 0.68	96.88 ± 0.78	95.46 ± 0.64
CLPT	98.02 ± 0.60	98.43 ± 0.23	98.65 ± 0.38	97.88 ± 0.37	97.12 ± 0.57	97.00 ± 0.85	95.67 ± 0.88
PSPT	98.81 ± 0.12	98.88 ± 0.26	98.90 ± 0.28	98.25 ± 0.62	97.89 ± 0.82	97.78 ± 0.36	96.43± 0.30

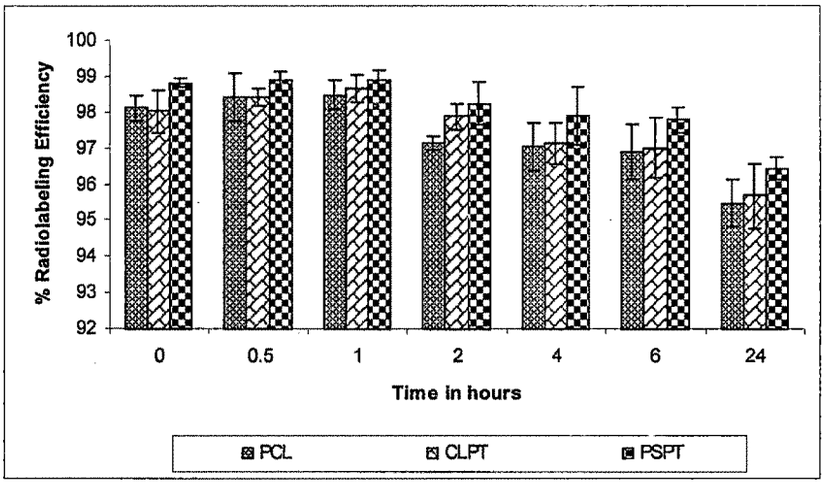

Figure 7.5. Stability study of radiolabeled PCL and its liposomal formulations in serum.

Table 7.6. DTPA challenging test of PCL and its liposomal formulations

Conc. of DTPA (mM)	% Transchelation (± S.E.)		
	PCL	CLPT	PSPT
25	3.78 ± 0.32	2.67 ± 0.12	1.36 ± 0.42
50	6.23 ± 0.60	4.88 ± 0.66	2.89 ± 0.40
75	8.72 ± 0.76	6.14 ± 0.35	5.70 ± 1.08
100	10.56 ± 1.02	8.08 ± 0.98	7.67 ± 0.96

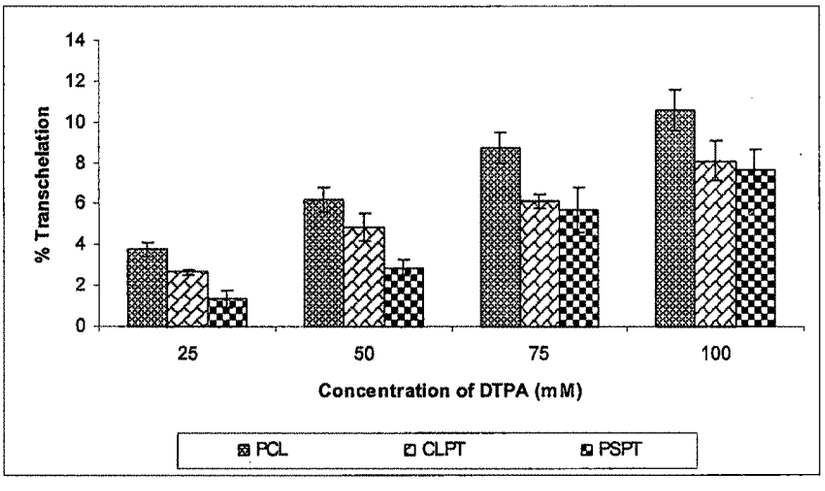

Figure 7.6. DTPA challenging test of PCL and its liposomal formulations

Table 7.7. Cysteine challenging test of PCL and its liposomal formulations

Conc. of Cysteine (mM)	% Transchelation (± S.E.)		
	PCL	CLPT	PSPT
25	1.76 ± 0.64	1.41 ± 0.39	1.01 ± 0.31
50	2.54 ± 0.91	2.28 ± 0.44	2.13 ± 0.52
75	4.43 ± 0.78	4.30 ± 0.6 4	4.01 ± 0.72
100	6.33 ± 0.97	6.00 ± 1.12	5.11 ± 0.44

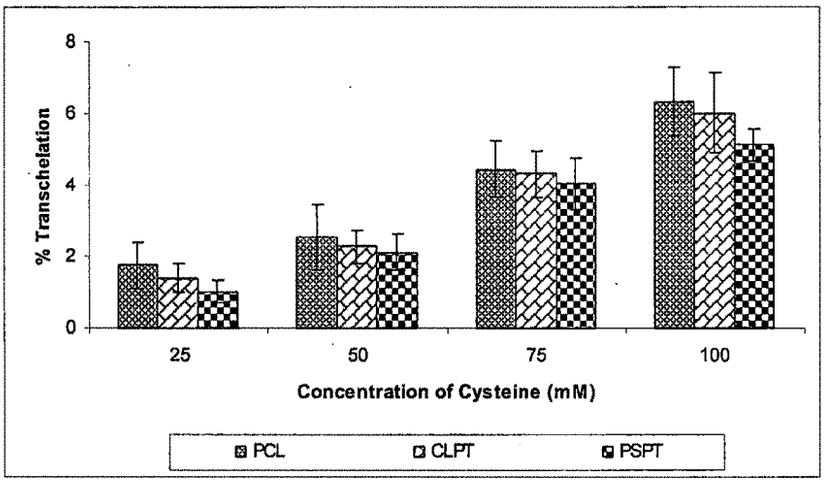

Figure 7.7. Cysteine challenging test of PCL and its liposomal formulations

7.5 Optimization of radiolabeling of Irinotecan and its liposomal formulation

The radiolabeling of Irinotecan Hydrochloride (IH) and its liposomal formulations was done with Technetium-99m by simple reduction method as described earlier.

Table 7.8. Effect of pH on radiolabeling efficiency of Irinotecan and its liposomal formulations

pH	Percent radiolabeled (± S.E)		
	IH	CLIH	PSIH
5.0	76.27 ± 1.25	76.87 ± 2.61	NA
5.5	78.43 ± 2.08	79.45 ± 2.33	NA
6.0	90.25 ± 1.06	93.27 ± 1.52	94.61 ± 1.82
6.5	96.44 ± 1.77	96.31 ± 2.24	97.13 ± 1.45
7.0	98.36 ± 1.43	98.44 ± 1.29	98.52 ± 0.94
7.5	97.31 ± 2.09	98.03 ± 1.42	98.15 ± 1.05
8.0	97.21 ± 1.96	97.78 ± 1.39	97.76 ± 1.26

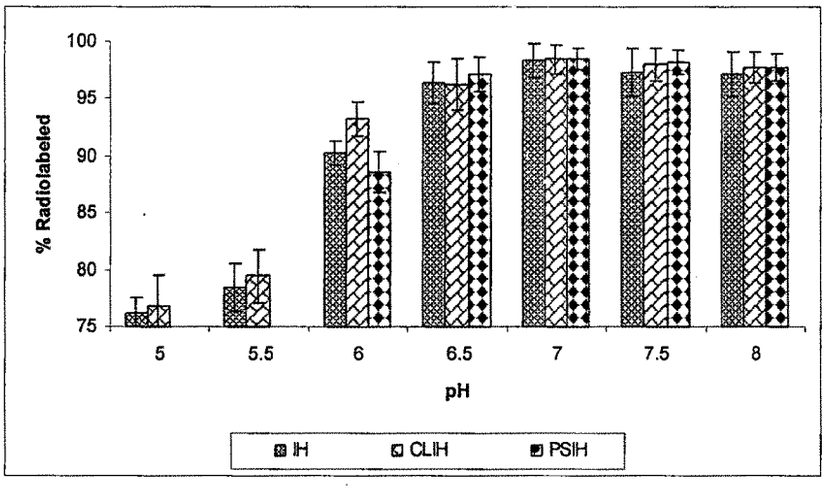

Figure 7.8. Effect of pH on radiolabeling efficiency of Irinotecan and its liposomal formulations

Table 7.9. Effect of incubation time on radiolabeling efficiency of Irinotecan and its liposomal formulations

Incubation time (min)	Percent radiolabeled (±S.E)		
	IH	CLIH	PSIH
0	84.12 ± 2.02	87.42 ± 2.65	87.66 ± 2.92
10	93.84 ± 2.66	95.88 ± 2.11	95.29 ± 1.64
20	96.19 ± 2.54	96.73 ± 1.46	97.43 ± 1.88
30	97.90 ± 1.84	98.10 ± 1.68	98.45 ± 1.48
45	98.22 ± 1.17	98.40 ± 1.04	98.52 ± 1.03
60	98.76 ± 1.06	98.75 ± 0.92	98.78 ± 0.77

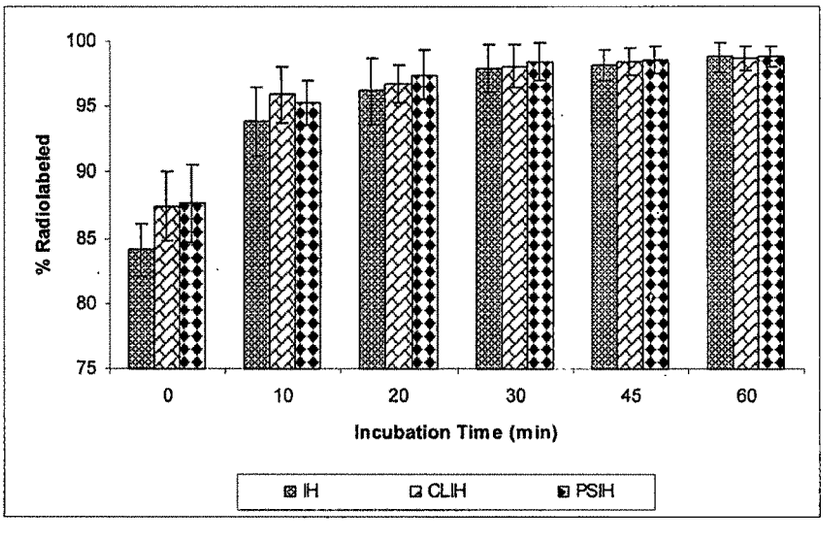

Figure 7.9. Effect of incubation time on radiolabeling efficiency of Irinotecan and its liposomal formulations

Table 7.10. Effect of $SnCl_2.2H_2O$ on radiolabeling efficiency of Irinotecan and its liposomal formulations

Conc. Of $SnCl_2.2H_2O$ (µg)	IH			CLIH			PSIH		
	% Bound	% Colloid	% Free	% Bound	% Colloid	% Free	% Bound	% Colloid	% Free
25	80.40	1.08	18.52	85.13	0.76	14.11	85.55	0.70	13.75
50	82.67	1.27	16.06	88.83	1.06	10.11	89.43	1.01	9.56
75	90.04	1.74	8.22	93.86	1.23	4.91	92.50	1.13	6.37
100	97.30	1.88	0.82	98.02	1.50	0.48	98.31	0.74	0.95
150	93.65	4.68	1.67	94.52	4.53	0.95	92.70	6.01	1.29
200	90.43	8.06	1.51	90.18	8.26	1.56	90.45	7.72	1.83

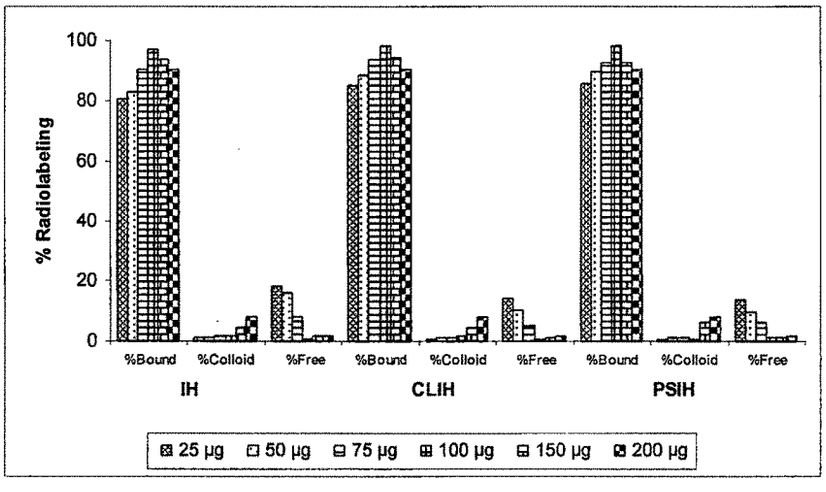

Figure 7.10. Effect of $SnCl_2.2H_2O$ on radiolabeling efficiency of Irinotecan and its liposomal formulations.

Table 7.11. Stability study of radiolabeled Irinotecan and its liposomal formulations in saline.

Formulations	% Radiolabeling Efficiency (± S.E.)						
	0 min	30 min	1 h	2 h	4 h	6 h	24 h
IH	98.45 ± 0.34	98.20 ± 0.43	98.54 ± 0.50	97.74 ± 0.48	97.68 ± 0.54	97.32 ± 1.04	95.06 ± 1.23
CLIH	98.48 ± 0.28	98.26 ± 0.92	98.62 ± 0.84	97.81 ± 0.76	97.68 ± 0.69	97.38 ± 0.90	95.58 ± 1.02
PSIH	98.51 ± 0.40	98.58 ± 0.92	98.82 ± 0.65	98.04 ± 0.61	97.75 ± 0.74	97.46 ± 1.02	95.81 ± 0.58

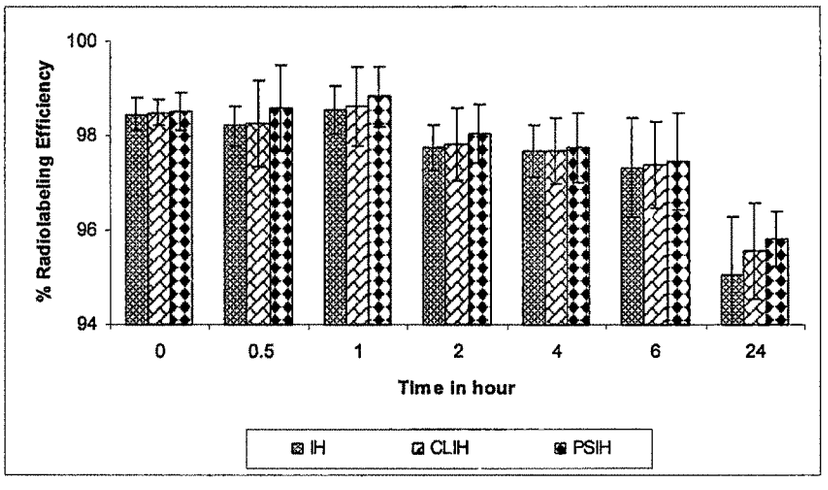

Figure 7.11. Stability study of radiolabeled Irinotecan and its liposomal formulations in saline.

Table 7.12. Stability study of radiolabeled Irinotecan and its liposomal formulations in serum

Formulations	% Radiolabeling Efficiency (± S.E.)						
	0 min	30 min	1 h	2 h	4 h	6 h	24 h
IH	97.84± 0.63	98.12 ± 0.46	98.33 ± 0.52	97.82± 0.73	97.44 ± 0.86	96.64 ± 1.10	95.26 ± 1.24
CLIH	98.11 ± 0.43	98.20 ± 0.67	98.16 ± 0.32	97.64 ± 0.58	97.05 ± 0.64	96.70 ± 0.90	95.35 ± 1.23
PSIH	98.43 ± 0.56	98.48 ± 0.64	98.24 ± 0.46	97.86 ± 0.62	97.28 ± 0.88	97.06 ± 1.21	95.43± 1.12

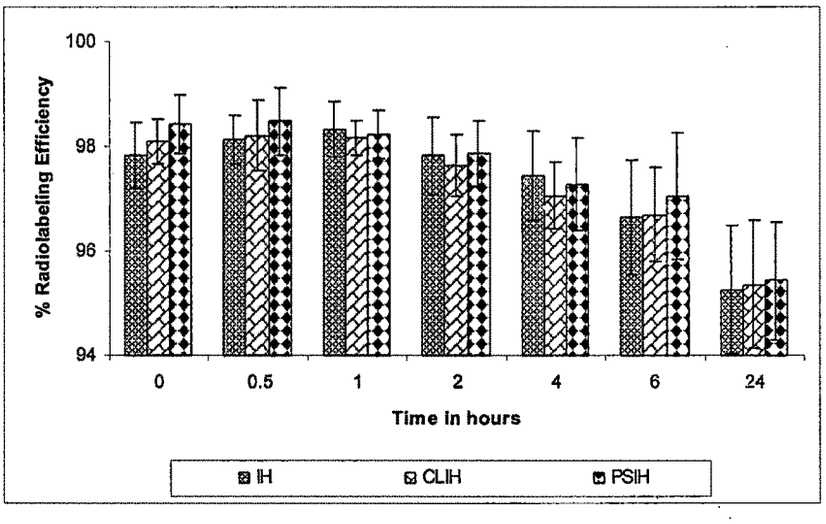

Figure 7.12. Stability study of radiolabeled Irinotecan and its liposomal formulations in serum.

Table 7.13. DTPA challenging test of Irinotecan and its liposomal formulations

Conc. of DTPA (mM)	% Transchelation (± S.E.)		
	IH	CLIH	PSIH
25	2.54 ± 0.86	2.45 ± 0.19	2.03 ± 0.58
50	4.86 ± 0.94	4.42 ± 0.62	3.64 ± 0.80
75	7.32 ± 0.70	6.78 ± 0.76	5.62 ± 0.98
100	8.18 ± 1.04	7.56 ± 1.03	6.68 ± 0.48

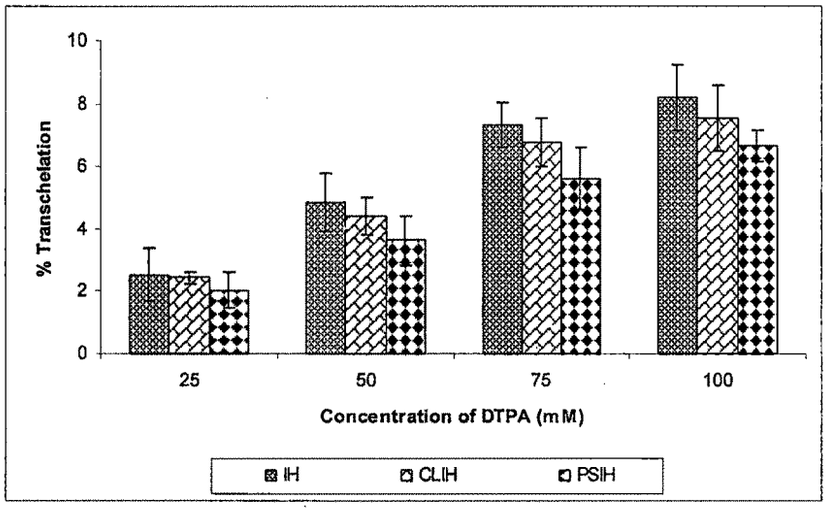

Figure 7.13. DTPA challenging test of Irinotecan and its liposomal formulations

Table 7.14. Cysteine challenging test of Irinotecan and its liposomal formulations

Conc. of Cysteine (mM)	% Transchelation (± S.E.)		
	IH	CLIH	PSIH
25	1.84 ± 0.66	1.46 ± 0.29	1.08 ± 0.32
50	2.98 ± 0.90	2.46 ± 0.43	2.26 ± 0.62
75	4.89 ± 0.75	4.41 ± 0.74	4.02 ± 0.78
100	6.48 ± 0.94	6.01 ± 1.03	5.23 ± 0.48

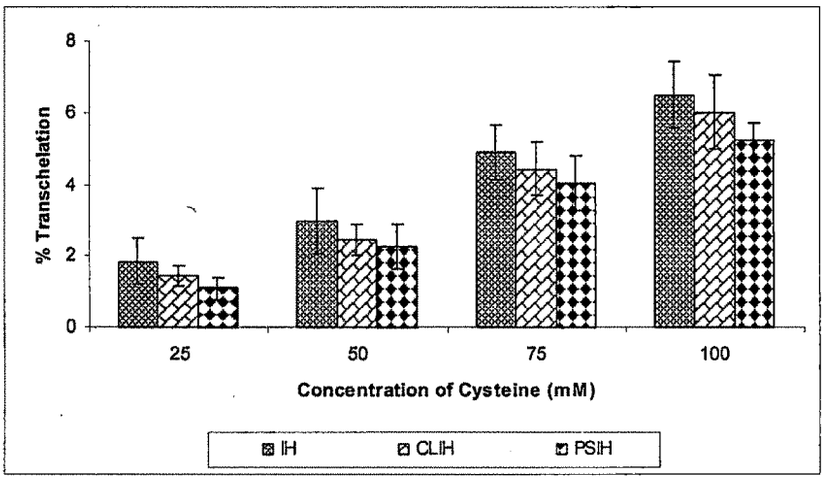

Figure 7.14. Cysteine challenging test of Irinotecan and its liposomal formulations.

7.6 RESULTS AND DISCUSSION

Radiolabeling of PCL and its liposomal formulations.

The PCL and its liposomal formulations were radiolabeled with high efficiency by the direct labeling technique using reduced ^{99m}Tc. The radiolabeling efficiency of ^{99m}Tc with PCL and its liposomal formulations was studied by ascending thin layer chromatography using ITLC-SG strips. The radiolabeling was optimized by taking three factors into account. i.e. pH of the complex, incubation time and stannous chloride dihydrate ($SnCl_2$. $2H_20$) concentration.

pH of the complex

The radiolabeling was carried out at various pH from 5 to 8 (pH 6 to 8 in case of PSPT) and the labeling efficiency was calculated (Table 7.1 and Figure 7.1). It was found that pH plays an important role in determining the labeling efficiency. As the pH increases from 5 to 7 the radiolabeling also increases from 70.34 % to 97.96 % for PCL and 71.45 % to 98.38 % for CLPT. In case of PSPT it increased from 90.77 % to 98.64%, when pH was raised from 6 to 7. Further increase in the pH led to reduction in the labeling efficiency. The optimum pH for labeling the PCL and its liposomal formulations was found to be 7 where excellent labeling took place.

Incubation time.

To find out the relationship between the incubation time and radiolabeling efficiency, the PCL and its liposomal formulations were mixed with the reduced ^{99m}Tc and incubated at various time intervals. The labeling efficiency was calculated after each time point (Table 7.2). The Figure 7.2 shows the effect of incubation time on labeling efficiency. The incubation time required for maximum labeling efficiency was found to be 30 min for PCL, CLPT and PSPT. Further increase in incubation time does not increase the labeling efficiency considerably.

$SnCl_2.2H_2O$ concentration

The concentration of $SnCl_2.2H_2O$ was found to be very critical in optimization of radiolabeling process. At low concentration of $SnCl_2.2H_2O$ the labeling of the compound was not complete. This led to the presence of free ^{99m}Tc, which was assessed by ITLC using 100 % acetone or 0.9 % saline as mobile phase. The Table 7.3 illustrates the effect of various concentrations of stannous chloride on labeling efficiency. By varying the amount of stannous chloride from 25 to 200 µg, but keeping the other factors constant at pH 7.0 and incubation time for 1 h, the influence on labeling yield was found to be significant. The labeling efficiency was increased from 84.57 % to 97.98% for PCL, 85.23 % to 98.09 % for CLPT and 85.76 % to 98.84% for PSPT with increase in stannous chloride amount from 25 to 100 µg. Further increase in the amount of stannous chloride lead to a reduction in yield and increase in concentration of reduced / hydrolyzed ^{99m}Tc. Thus the optimum concentration of $SnCl_2.2H_2O$ was found to be 100 µg for efficient radiolabeling.

Stability studies of ^{99m}Tc-labeled PCL and its liposomal formulations.

Stability of the ^{99m}Tc-labeled complexes with time was studied in saline and in serum (rabbit) at 37°C as shown in Table 7.4 and Table 7.5 respectively. The experimental data revealed that there was hardly any detachment of radioisotope from the complex. Even after 24 h incubation, the presence of more than 95 % labeled complex and only 3-5 % decrease in labeled product signifies the high stability of the radiolabeled complex and its suitability for *in-vivo* use.

High binding affinity of the ^{99m}Tc with PCL and its liposomal formulations was established by incubating the labeled compound with DTPA and Cysteine at different molar concentrations from 25 to 100mM as shown in Table 7.6, Table 7.7 and Figure 7.6, Figure 7.7 respectively. The percent transchelation of the ^{99m}Tc- PCL / liposomal formulations was found to be less than 4.0 % at 25 mM concentration of DTPA and cysteine. Even at high concentration of 100mM the maximum transchelation was found to be only around 10 % proves the strength and stability of the ^{99m}Tc- PCL / liposomal complex.

Radiolabeling of Irinotecan and its liposomal formulations.

The Irinotecan and its liposomal formulations were radiolabeled with high efficiency by the direct labeling technique using reduced ^{99m}Tc. The radiolabeling efficiency of ^{99m}Tc with Irinotecan and its liposomal formulations was studied by ascending thin layer chromatography using ITLC-SG strips. The radiolabeling was optimized by taking three factors into account. i. e. pH of the complex, incubation time and stannous chloride dihydrate concentration.

pH of the complex

The radiolabeling was carried out at various pH from 5 to 8 (6 to 8 for PSIH) and the labeling efficiency was calculated (Table 7.8 and Figure 7.8). It was found that pH plays an important role in determining the labeling efficiency. As the pH increased from 5 to 7 the radiolabeling also increased from 76.27 % to 98.36 % for Irinotecan and 76.87 % to 98.44 % for CLIH. In case of PSIH it increased from 88.61 % to 98.52 % when pH was raised from 6 to 7. Further increase in the pH led to reduction in the labeling efficiency. The optimum pH for labeling the Irinotecan and its liposomal formulations was found to be 7 where excellent labeling took place.

Incubation time

To find out the relationship between the incubation time and radiolabeling efficiency, the Irinotecan and its liposomal formulations were mixed with the reduced ^{99m}Tc and incubated at various time intervals. The labeling efficiency was calculated after each time point (Table 7.9). The figure 7.9 shows the effect of incubation time on labeling efficiency. The incubation time required for maximum labeling efficiency was found to be 30 min for Irinotecan, CLIH and PSIH. Further increase in incubation time does not increase the labeling efficiency considerably.

$SnCl_2.2H_2O$ concentration

The concentration of $SnCl_2.2H_2O$ was found to be very critical in optimization of radiolabeling process. At low concentration of $SnCl_2.2H_2O$ the labeling of the compound was not complete. This led to the presence of free ^{99m}Tc, which was assessed by ITLC using 100 % acetone or 0.9 % saline as mobile phase. The table 7.10 illustrates the effect of various concentrations of stannous chloride on labeling efficiency. By varying the amount of stannous chloride from 25 to 200 µg, but keeping the other factors constant at pH 7.0 and incubation time for 1 h, the influence on labeling yield was found to be significant. The labeling efficiency increased from 80.40 % to 97.30 % for Irinotecan, 85.13 % to 98.02 % for CLIH and 85.55 % to 98.31 % for PSIH with increase in stannous chloride amount from 25 to 100 µg. Further increase in the amount of stannous chloride leads to a reduction in yield and increase in concentration of reduced / hydrolyzed ^{99m}Tc. Thus the optimum concentration of stannous chloride dihydrate was found to be 100 µg for efficient radiolabeling.

Stability studies of ^{99m}Tc-labeled Irinotecan and its liposomal formulations.

Stability of the ^{99m}Tc-labeled complexes with time was studied in saline and in serum (rabbit) at 37°C as shown in Table 7.11 and Table 7.12 respectively. The experimental data revealed that there was hardly any detachment of radioisotope from the complex. Even after 24 h incubation, the presence of more than 95 % labeled complex and only 3-5 % decrease in labeled product signifies the high stability of the radiolabeled complex and its suitability for *in-vivo* use.

High binding affinity of the ^{99m}Tc with Irinotecan and its liposomal formulations was established by incubating the labeled compound with DTPA and Cysteine at different molar concentrations from 25 to 100mM as shown in Table 7.13, 7.14 and figure 7.13, 7.14. The percent transchelation of the ^{99m}Tc- Irinotecan / liposomal formulations was found to be less than 3.0 % at 25 mM concentration of DTPA and cysteine. Even at high concentration of 100 mM the maximum transchelation was found to be only around 8 % proves the strength and stability of the ^{99m}Tc- Irinotecan / liposomal complex.

7.7 REFERENCES

Babbar, A. K., Sharma, R.K. (2003) Hospital Radiopharmacy: part IV-Formulation, quality control and dispensing issues. *Ind J Hosp Pharm.*, Jan-Feb: 8-14.

Barratt, G.M., Tuzel, N.S., Ryman, B.E. (1984) The labeling of liposomal membranes with radioactive technetium, In: Liposome Technology: Vol 1, G. Gregoriadis (Ed.) CRC press, Boca Raton, FL. 93-106.

Budinger, T. (1980) Physical attributes of single–photon tomography. *J. Nucl. Med.* **21**: 579-592.

Chauhan, U.P.S., Mishra, P., Chander, J. (1993) ^{99m} Tc-Diethyl monoiodo- IDA: a radiopharmaceutical for hepatobiliary scintigraphy. *Appl Radiat Isot.* **44**: 843-848.

Eckelman, W.C., Park, C.H., Steigman, J. (1989) Three approaches to radiolabeling antibodies with ^{99m} Tc. *Nucl. Med. Biol.* **16**: 171-176.

Gulati, M., Singh, N., Singh, A. K., Chopra, M. K., Bhatnagar, A., Agrawal, S.S. (2005) Development and Potentials of Tc-99m Salbutamol (Saltec). *Ind. J. Nucl. Med.* **20**: 72-76.

Mishra, A.K., Iznaga Escobar, N., Figuerodo, R., Jain, V. K., Dwarakanath, B. S., Rodriguez, P., Sharma, R. K., Lazar Mathew, T. (2002) Preparation and comparative evaluation of ^{99m} Tc labeled 2-Iminotholane modified antibodies and CITC-DTPA immunoconjugates of anti-EGF-receptor antibodies. Methods Find. Exp. Clin. Pharmacol., **24**: 653-660.

Mishra, P., Chuttani, K., Mishra, A. K., Sharma, R.K. (1999) Radiolabeling of diltiazem with ^{99m} Tc and its evaluation for tumor targeting. *Ind J Nucl Med.*, **14**: 249-254.

Ogihara, J., Kojima, S., Jay, M. (1986) Differential uptake of Ga-67 labeled liposomes between tumours and inflammatory lesions in rats. *J. Nucl. Med.* **27**: 1300-1307.

Presne, C.A., Proffitt. R.T., Williams, L.E., Winsor, D., Wernerr, J.L., Kennedy, P., Wiseman, C., Gala, K., Mckenna, R.J., Smith, J.D., Bouzaglou, S.A., Callahan, R.A., Baldeschweiler, J., Crosseley, R.J. (1989) Successful imaging of human cancer with Indium-111 labeled phospholipids vesicles. *Cancer,* **46**: 951-958.

Reddy, L. H., Sharma, R.K., Chuttani, K., Mishra, A. K., Murthy, R.S.R. (2004) Etoposide-incorporated Tripalmitin Nanoparticles With Different Surface Charge: Formulation, Characterization, Radiolabeling, and Biodistribution Studies. *AAPSJ.* **6**: article 23.

Richardson, V.J., Jeyasingh, K., Jewkes, R.F. (1977) Properties of [^{99m} Tc] technetium labeled liposomes in normal and tumor bearing rats. *Biochem Soc. Trans.* **5**: 290-291.

Saha, G.B. (1993) Physics and radiobiology of nuclear medicine. Springer- Verlag, NewYork; 98-156.

Sorenson JA, Phelps ME. Physics in nuclear medicine. Second ed. (1987) Grune and Stratton, Inc. 262-276.

Theobald, A.E., In: Sampson CB. (ed.): Text book of Radiopharmacy: Theory and Practice. (1990) Gorden and Breach, NY. Chapter 7, 127-135.