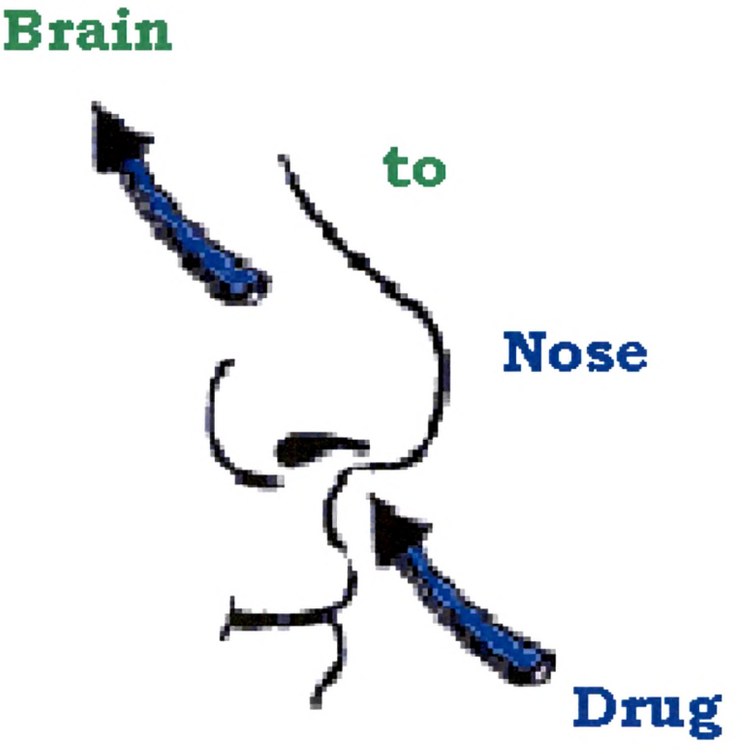

CHAPTER 6

RADIOLABELING OF FORMULATIONS

6.1 Radiolabeling of Formulations

6.1.1 Radiolabeling of Tacrine Solution, Microemulsion and Mucoadhesive Microemulsion:

The tacrine solution (TS), tacrine microemulsion (TME) and tacrine mucoadhesive microemulsion (TMME) were labeled using ^{99m}Tc by direct labeling method (Eckelman et al 1995; Babbar et al 2000; Mishra et al 2004). One mL of either TS (33mg/mL) or TME (33mg/mL) or TMME (33mg/mL) was taken. To this solution, 200 µg of stannous chloride dehydrate ($SnCl_2$, $2H_2O$) in 100 µL of 10% v/v acetic acid was added and pH was adjusted to 6.5 ± 0.2 using 0.5 M sodium bicarbonate solution. To the resultant mixture (filtered through 0.22 µm nylon 66 membrane), 1 mL of sterile sodium ^{99m}Tc-pertechnatate (75 to 400 MBq) was added drop wise over a period of 60 seconds with continuous mixing. The mixture was incubated at room temperature for 30 min with continuous nitrogen purging. The final volume was made up to 2.50 mL using 0.90% w/v sodium chloride solution (Babbar et al. 2000).

The radiochemical purities of ^{99m}Tc-TS, ^{99m}Tc-TME and ^{99m}Tc-TMME were assessed using ascending instant thin layer chromatography (ITLC). Silica gel (SG) - coated fiber glass sheets (Gelman Sciences Inc., Ann Arbor, MI, USA) and dual solvent systems consisting of acetone and pyridine: acetic acid: water (3: 5: 1.50 v/v) were employed as stationary and mobile phases respectively (Saha 1993; Saha 2005). Approximately 2 to 3 µL of the radio labeled complex was applied approximately at a point 1 cm from one end of ITLC-SG strip (Theobald 1990). The strip was developed using acetone and pyridine: acetic acid: water. The solvent front was allowed to run up to 8 cm from the point of application. The strip was cut horizontally in to two halves. The radioactivity in each segment was estimated in a well-type gamma ray counter (Gamma ray spectrometer, type GRS23C, Electronics Corporation of India Ltd, Mumbai, India). The radiolabeled complex and the contaminants like reduced/hydrolyzed (R/H) ^{99m}Tc and free ^{99m}Tc-pertechnetate were estimated. Migration values (R_f) for free ^{99m}Tc, R/H ^{99m}Tc and ^{99m}Tc-tacrine are mentioned in Table 6.1.

Table 6.1 Migration values (R_f) of ^{99m}Tc-pertechnetate, reduced/hydrolyzed (R/H) ^{99m}Tc and ^{99m}Tc-tacrine determined using ascending ITLC (SG) and two different solvent systems

Solvent system	R_f value		
	Free ^{99m}Tc	R/H ^{99m}Tc	^{99m}Tc-tacrine
Acetone	0.90	0.00	0.00
Pyridine: acetic acid: water	0.90	0.00	0.90

Optimization of Radiolabeling

The radiolabeling was optimized by taking three factors in consideration (1) incubation time (2) pH of the complex, and (3) concentration of $SnCl_2.2H_2O$. The labeled complexes were incubated for 15, 30, 45 and 60 min to evaluate optimum incubation time for maximum labeling efficiency and the results are shown in Table 6.2. The pH was adjusted ranging from 5.5 to 7.5 and the outcome on labeling efficiency was studied and the results are shown in Figure 6.1. The effect of $SnCl_2.2H_2O$ concentration was also studied in order to arrive at optimal concentration for maximum labeling (Theobald 1990; Saha 1993; Saha 2005) and the results are shown in Figure 6.2.

Table 6.2 Effect of incubation time on the labeling efficiency of ^{99m}Tc-TS/TME/TMME.

Incubation time (min)	% Radiolabeled*		
	^{99m}Tc-tacrine solution (^{99m}Tc-TS)	^{99m}Tc-tacrine microemulsion (^{99m}Tc-TME)	^{99m}Tc-tacrine mucoadhesive microemulsion (^{99m}Tc-TMME)
0	78.46 ± 3.53	77.24 ± 2.01	75.45 ± 2.56
15	84.56 ± 1.63	86.33 ± 1.54	85.44 ± 2.03
30	96.35 ± 1.24	97.66 ± 1.06	98.31 ± 1.57
45	95.77 ± 1.16	97.15 ± 1.28	97.86 ± 1.01
60	95.54 ± 1.05	97.03 ± 1.20	97.65 ± 1.04

*The results are mean ± SD of three separate experiments.

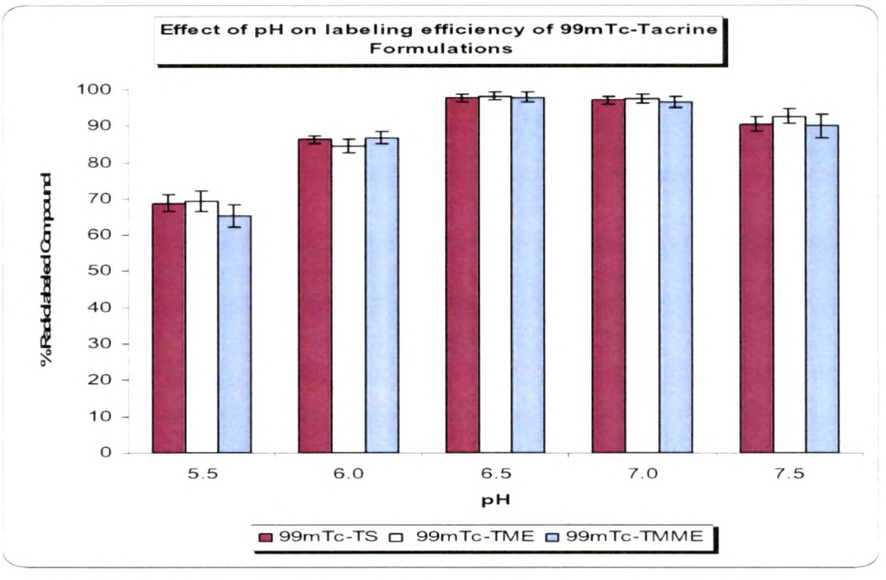

Figure 6.1 Effect of pH on labeling efficiency of ^{99m}Tc-TS/TME/TMME. Results are the mean of three separate experiments. Error bar represents SD.

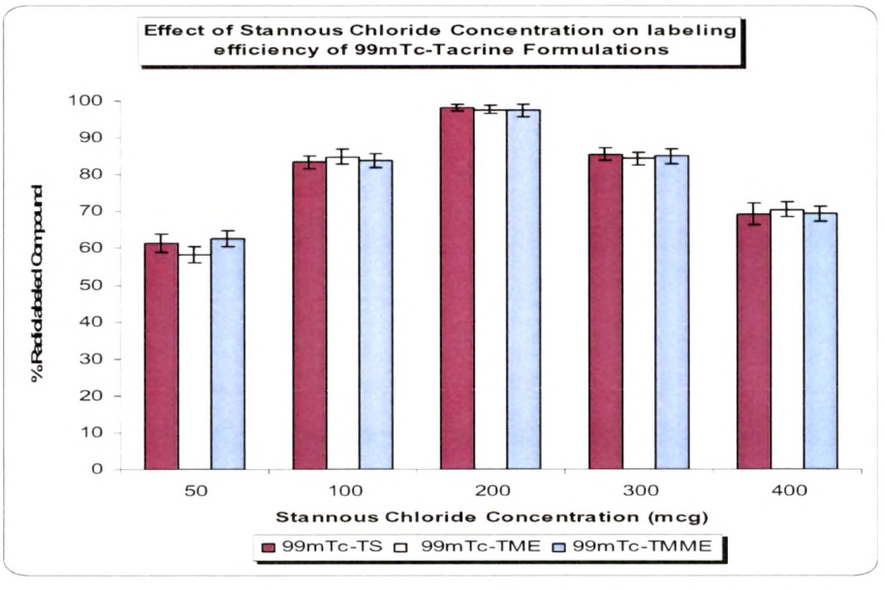

Figure 6.2 Effect of stannous chloride concentration on labeling efficiency of ^{99m}Tc-TS/TME/TMME. Results are the mean of three separate experiments. Error bar represents SD.

***In Vitro* Stability of ^{99m}Tc-tacrine solution/microemulsion/mucoadhesive microemulsion**

The stability studies of ^{99m}Tc-TS/TME/TMME were carried out *in vitro* using 0.90% w/v sodium chloride and mice serum by ascending ITLC (Garron et al. 1991). For *in vitro* stability, 100 µL of radiolabel complex was mixed in triplicate with 2.40 mL 0.90% w/v sodium chloride (physiological saline) and mice serum each. ITLC was performed after incubating at 37°C at predetermined time intervals up to 24 h to assess the labeling efficiency. The results are given in Table 6.3.

Transchelation challenge test using diethylene triamine penta acetic acid

To evaluate stability and bonding strength of the radio labeled solution/microemulsion/mucoadhesive microemulsion, one mL of the radio labeled formulation was challenged against various concentrations (25, 50, 75 and 100 mM) of diethylene triamine penta acetic acid (DTPA) (Babbar et al. 2000; Saha 1993; Saha 2005). The mixtures were incubated for 4 h at 37°C and the labeling efficiency was measured using ITLC-SG coated glass sheets as stationary phase and acetone and pyridine: acetic acid: water (3: 5: 1.50 v/v) systems as mobile phases. Approximately 2 to 3 µL complex was applied at 1 cm distance on the ITLC-SG and mobile phase was allowed to run up to 8 cm from the point of application. The separated pertechnetate and DTPA complex were determined at migration value 0.90 (R_f= 0.90) while ^{99m}Tc-TS/TME/TMME remained at the point of application (R_f = 0). Effect of different molar concentrations and percent transchelation is illustrated in Figure 6.3.

Table 6.3 *In vitro* stability of ^{99m}Tc-TS/TME/TMME in 0.90% w/v sodium chloride and mice serum at 37°C*

Time (h)	% Radiolabeled					
	In 0.90% w/v Sodium Chloride			In Mice Serum		
	TS	TME	TMME	TS	TME	TMME
0.50	97.64 ± 1.09	97.76 ± 1.05	98.12 ± 1.23	97.88 ± 1.03	97.92 ± 1.14	98.28 ± 1.08
1	97.48 ± 1.06	97.65 ± 1.07	98.54 ± 1.22	97.92 ± 1.08	98.29 ± 1.22	98.39 ± 1.00
2	97.16 ± 1.04	97.03 ± 1.42	98.19 ± 1.35	97.62 ± 1.05	97.79 ± 1.37	97.68 ± 1.16
4	96.98 ± 1.01	96.87 ± 1.49	97.82 ± 1.24	96.97 ± 1.07	97.21 ± 1.21	97.34 ± 1.13
6	96.17 ± 1.12	96.51 ± 1.53	96.61 ± 1.16	96.43 ± 1.26	96.49 ± 1.41	96.75 ± 1.30
24	95.88 ± 1.12	95.11 ± 1.43	95.22 ± 1.33	95.68 ± 1.32	95.34 ± 1.16	95.73 ± 1.20

* Data are expressed in terms of percentage of total radioactivity in sample. Results are mean ± SD of three separate experiments.

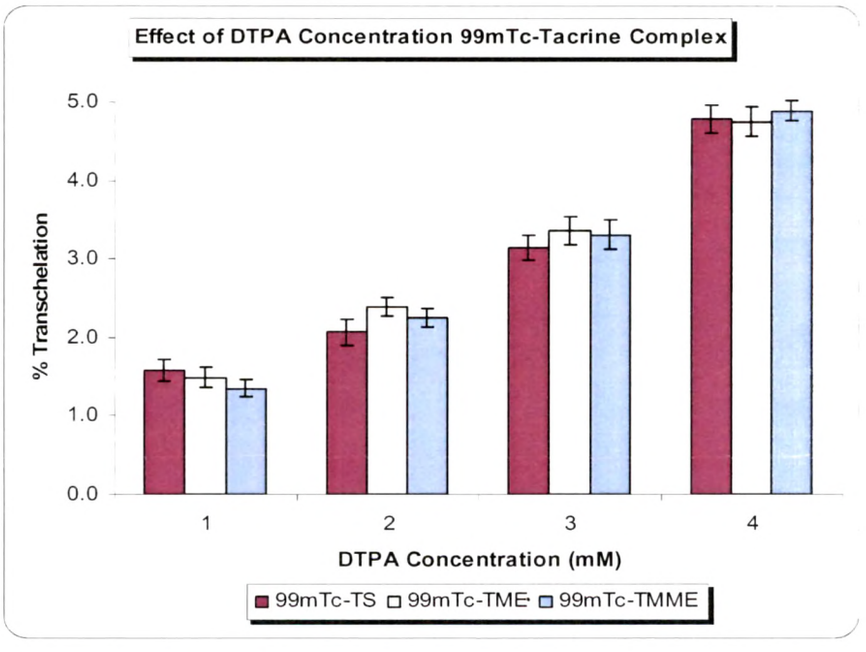

Figure 6.3 Effect of variable molar concentrations of DTPA on radiolabeled ^{99m}Tc-tacrine complexes. Percentage transchelation was measured using ITLC-SG. Results are mean of three separate experiments. Error bar represents SD.

6.1.2 Radiolabeling of Donepezil Solution, Microemulsion, and Mucoadhesive microemulsion:

The donepezil solution (DS), donepezil microemulsion (DME) and donepezil mucoadhesive microemulsion (DMME) were labeled using ^{99m}Tc by direct labeling method (Eckelman et al 1995; Babbar et al 2000; Mishra et al 2004). One mL of either DS (16.67mg/mL) or DME (16.67mg/mL) or DMME (16.67mg/mL) was taken. To this solution, 200 µg of stannous chloride dihydrate in 100 µL of 10% v/v acetic acid was added and pH was adjusted to 6.5 ± 0.2 using 0.5 M sodium bicarbonate solution. To the resultant mixture (filtered through 0.22 µm nylon 66 membrane), 1 mL of sterile sodium ^{99m}Tc-pertechnatate (75 to 400 MBq) was added drop wise over a period of 60 seconds with continuous mixing. The mixture was incubated at room temperature for 30 min with continuous nitrogen purging. The final volume was made up to 2.50 mL using 0.90% w/v sodium chloride solution (Babbar et al. 2000).

The radiochemical purities of ^{99m}Tc-DS, ^{99m}Tc-DME and ^{99m}Tc-DMME were assessed using ascending ITLC as mentioned in section 6.1.1. Migration values (R_f) for free ^{99m}Tc, R/H ^{99m}Tc and ^{99m}Tc-donepezil are mentioned in Table 6.4. The effect of incubation time, pH and stannous chloride concentration on labeling efficiency were studied to achieve optimum reaction conditions as mentioned in section 6.1.1. The result of effect incubation time, pH and stannous chloride concentration is recorded in Table 6.5, Figure 6.4 and Figure 6.5 respectively. The optimized radiolabeled formulations were assessed for *in vitro* stability in 0.90% w/v sodium chloride (physiological saline) and in mice serum as mentioned in section 6.1.1. The results of stability in physiological saline and in mice serum are recorded in Table 6.6. The radiolabeled formulations were challenged for bonding strength using DTPA as mentioned in section 6.1.1. The effect of DTPA on transchelation is illustrated in Figure 6.6.

Table 6.4 Migration values (R_f) of ^{99m}Tc-pertechnetate, reduced/hydrolyzed (R/H) ^{99m}Tc and ^{99m}Tc-donepezil determined using ascending ITLC (SG) and two different solvent systems

Solvent system	R_f value		
	Free ^{99m}Tc	R/H ^{99m}Tc	^{99m}Tc-donepezil
Acetone	0.90	0.00	0.00
Pyridine: acetic acid: water	0.90	0.00	0.90

Table 6.5 Effect of incubation time on the labeling efficiency of ^{99m}Tc-DS/DME/DMME.

Incubation time (min)	% Radiolabeled*		
	^{99m}Tc- donepezil solution (^{99m}Tc-DS)	^{99m}Tc- donepezil microemulsion (^{99m}Tc-DME)	^{99m}Tc- donepezil mucoadhesive microemulsion (^{99m}Tc-DMME)
0	80.22 ± 2.08	79.82 ± 1.83	80.56 ± 2.11
15	86.36 ± 1.51	87.77 ± 1.35	86.56 ± 1.16
30	98.86 ± 1.19	98.42 ± 1.04	98.02 ± 1.20
45	98.22 ± 1.01	97.92 ± 1.25	97.68 ± 1.05
60	97.87 ± 1.14	97.69 ± 1.06	97.51 ± 1.09

*The results are mean ± SD of three separate experiments.

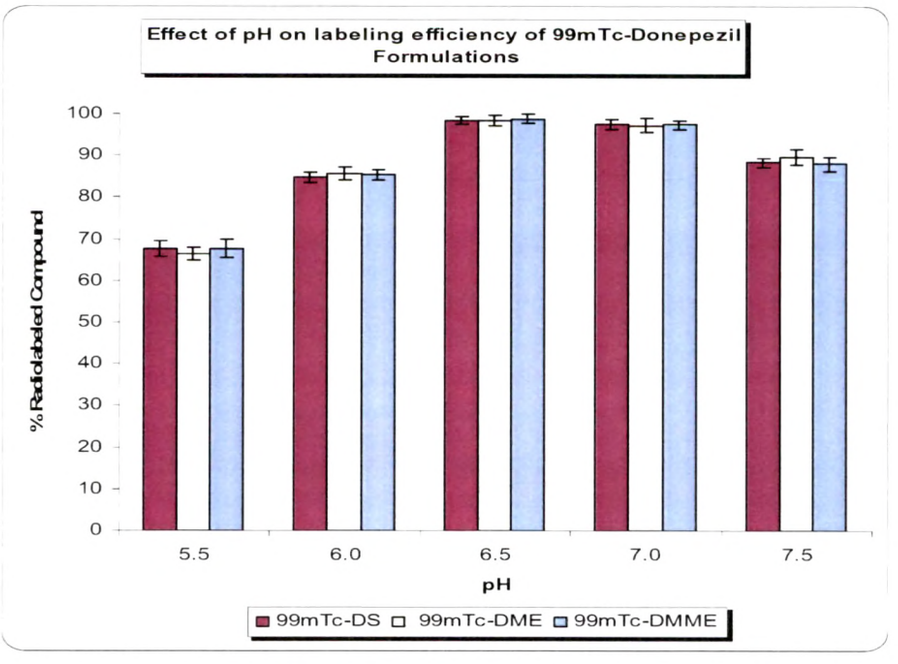

Figure 6.4 Effect of pH on labeling efficiency of ^{99m}Tc-DS/DME/DMME. Results are the mean of three separate experiments. Error bar represents SD.

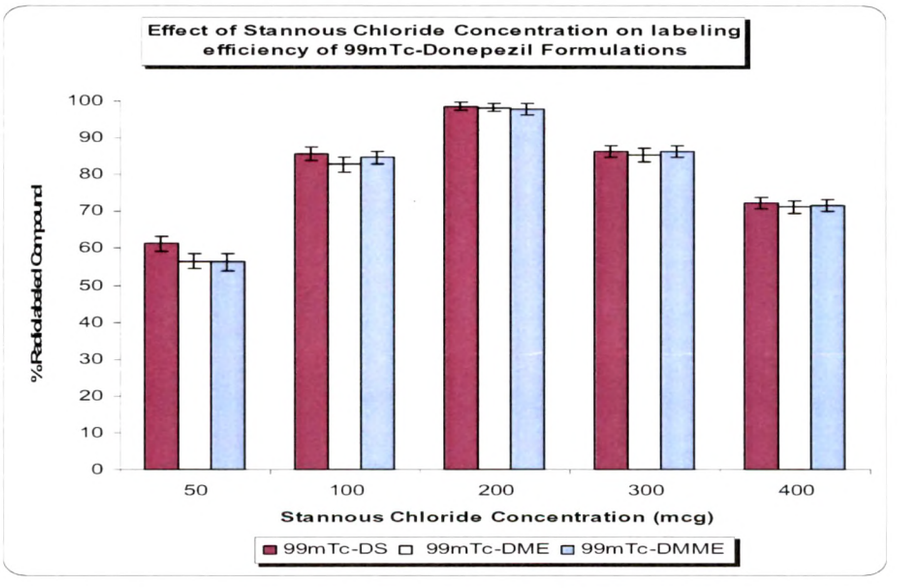

Figure 6.5 Effect of stannous chloride concentration on labeling efficiency of ^{99m}Tc-DS/DME/DMME. Results are the mean of three separate experiments. Error bar represents SD.

Table 6.6 *In vitro* stability of ^{99m}Tc-DS/DME/DMME in 0.90% w/v sodium chloride and mice serum at 37°C*

Time (h)	% Radiolabeled					
	In 0.90% w/v Sodium Chloride			In Mice Serum		
	DS	DME	DMME	DS	DME	DMME
0.50	98.64 ± 1.04	98.52 ± 1.50	98.18 ± 1.36	97.84 ± 1.58	98.62 ± 1.01	98.57 ± 1.36
1	97.92 ± 1.01	97.72 ± 1.20	97.60 ± 1.22	98.23 ± 1.27	98.14 ± 1.07	98.14 ± 1.38
2	98.12 ± 1.09	97.88 ± 1.16	98.02 ± 1.35	98.08 ± 1.38	97.86 ± 1.05	97.86 ± 1.32
4	97.44 ± 1.13	97.17 ± 1.17	97.42 ± 1.15	97.66 ± 1.34	97.28 ± 1.05	97.28 ± 1.02
6	96.56 ± 1.05	96.44 ± 1.34	96.84 ± 1.07	96.78 ± 1.02	96.11 ± 1.07	96.11 ± 1.32
24	95.31 ± 1.39	95.24 ± 1.72	95.67 ± 1.24	95.53 ± 1.09	95.16 ± 1.18	95.16 ± 1.21

* Data are expressed in terms of percentage of total radioactivity in sample. Results are mean ± SD of three separate experiments.

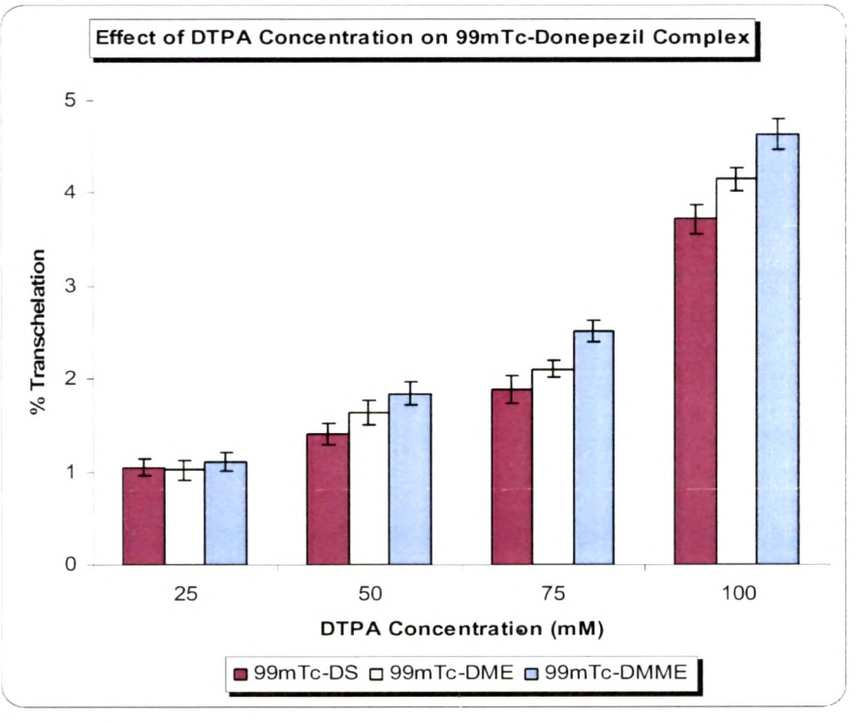

Figure 6.6 Effect of variable molar concentrations of DTPA on radiolabeled ^{99m}Tc-donepezil complex. Percentage transchelation was measured using ITLC-SG. Results are mean of three separate experiments. Error bar represents SD.

Table 6.7 Optimum reaction conditions for radiolabeling of tacrine and donepezil formulations

Sr. No.	Parameters	Tacrine Formulations	Donepezil Formulations
1	Radiolabeling efficiency	> 96.35%	> 98.02%
2	Ph	6.5 ± 0.2	6.5 ± 0.2
3	$SnCl_2.2H_2O$ (µg/mL)	200	200
4	Incubation time (min)	30	30
5	DTPA Challenge Test (transchelation % w/w at 100 mM DTPA concentration)	<5.00% w/w	<4.65% w/w
6	*In vitro* stability (After 24 h)	> 95%	> 95%

6.2 Results and Discussion

Tacrine formulations and Donepezil formulations were successfully radiolabeled using ^{99m}Tc direct labeling method (external labeling method). The radiolabeling was performed using the methods reported in literature (Eckelman et al 1995; Babbar et al 2000; Mishra et al 2004). However, the reaction conditions were optimized to achieve maximum radiolabeling efficiency (> 95%). Radiochemical purities achieved were 96.35%, 97.66% and 98.31% for TS, TME and TMME respectively when evaluated for R/H ^{99m}Tc and free ^{99m}Tc. The optimal $SnCl_2.2H_2O$ concentration was found to be 200 µg/mL at pH 6.5 ± 0.2 with an incubation time of 30 min. ^{99m}Tc-TS/TME/TMME were found to be stable in 0.90% w/v sodium chloride solution (physiological saline) and in mice serum up to 24 h (degradation < 5%w/w). Bonding strength of ^{99m}Tc-TS/TME/TMME was also investigated by the DTPA challenging test, and the percent transchelation of the labeled complex was <1.60% w/w at 25 mM DTPA concentration, while at 100 mM, it increased up to only around 5.00% w/w. The results suggested high bonding strength and stability of ^{99m}Tc-TS/TME/TMME. Thus, these formulations were found suitable for *in vivo* studies (Garron et al. 1991; Stein et al. 1999).

DS, DME and DMME were successfully radiolabeled with ^{99m}Tc by direct labeling method and optimized for maximum labeling efficiency and stability. The radiochemical purities achieved were 98.86%, 98.42%, and 98.02% for DS, DME, and DMME respectively when evaluated for R/H ^{99m}Tc and free ^{99m}Tc. The optimal $SnCl_2.2H_2O$ concentration was found to be 200 µg/mL at pH 6.5 ± 0.2 and incubation time of 30 min. The ^{99m}Tc labeled formulations were found to be stable in 0.90% w/v sodium chloride solution (physiological saline) and in mice serum up to 24 h (degradation < 5% w/w). Bonding strength of all ^{99m}Tc labeled formulations were also investigated by DTPA challenging test and the percent transchelation of the labeled complex was <1.15% w/w at 25 mM DTPA concentration, while at 100 mM, it increased up to only around 4.65% w/w. The results suggest high bonding strength and stability ^{99m}Tc labeled donepezil formulations and hence, were found suitable for *in vivo* studies (Garron et al. 1991; Stein et al. 1999).

The results of optimum radiolabeling conditions and critical parameters are summarized in Table 6.7 for tacrine and donepezil formulations used to study biodistribution.

6.3 References

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