CHAPTER 8

RADIOLABELING OF FORMULATIONS

8 RADIOLABELING OF FORMULATIONS

8.1 Introduction

Radiolabeling of drugs and drug delivery systems has been widely applied to study these biological distribution patterns. Particularly, the radiolabeling with short lived radionuclides has been preferred due to their rapid decay and hence low toxicity. Drugs or colloidal drug carriers are linked to the radionuclides that are tailored for preferable concentration by a particular organ or physiologic process. In practice, the majority of radiopharmaceuticals are used for diagnosis (Mishra P et al., 1999), but there a number of radionuclides available for the treatment of some disorders, especially cancer (Babbar AK and Sharma RK., 2003). In the typical radiopharmaceutical formulation, the quantities of radionuclides and pharmaceutical agent used are normally quite less. The radiopharmaceutical differs from the 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, function as a true tracer, and they are generally free from hypersensitivity reactions. Since the dose administered is very low, the control of parameters such as tonicity and pyrogenicity is also not so important. The natural decay process may result in change in the final radionuclide composition and in the degradation of the stable materials. Variation in quality of radiopharmaceutical can greatly affect the biodistribution pattern and thereby the ultimate scan quality, causing problems in interpretation.

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 AK and Sharma RK., 2003).

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 tracing using an external detection system viz. gamma camara (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 the distribution of radioactivity within certain organ system using emitted gamma rays upon administration of a radio tracer (Sorensen JA and Phelps ME., 1980; Budinger TF., 1980). Gamma imaging has lead to an increase in the demand for short lived radio tracers which can be safely administered in larger doses with minimal radiation dose. For biological experiments, the radionuclides are linked to the compounds of interest by various techniques. The effective binding of radiolabelled 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 radiolabel and serum stability.

In practice, the radiopharmaceutical preparation is administered to the species of interest, using by the parenteral route. At specified time intervals, the organs or tissues of interest are removed and measured for radioactivity using a gamma counter. The images of organs/tissues can also be taken without sacrificing the host using the SPECT camera. Various radionuclides are used for the above mentioned purposes include ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{99}Mo, ^{131}I, ^{123}I, ^{133}Xe, ^{201}Tl, ^{99m}Tc, ^{67}Ga, ^{111}In (Ramamoorthy N and Desai CN., 1997).

Various reports are available where ^{99m}Tc has been widely used for the pharmacokinetic and biodistribution studies of many drugs and their delivery systems. Technetium is prepared by the following reaction from Uranium (^{235}U)

$$^{235}U \xrightarrow{\text{Irradiated with neutron flux}} {}^{99}Mo \longrightarrow {}^{99m}Tc$$

Common methods of separation of ^{99m}Technicium and ^{99} Molybdenum

1. Column Chromatography over acidic alumina

2. Solvent extraction of ^{99m}Tc with methyl ethyl ketone
3. Sublimination of Tc oxides from Mo compounds

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) crystal. 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 photomultiplier tube, the intensity of light is enhanced and passes through a pre-amplifier and linear amplifier and consequently to the pulse height analyzer. The signals are then tuned in a tuner and recorded in the 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.

Physical Properties of ^{99m}Technicium

^{99m}Tc decays by isomeric transition with the physical half life of 6.02h. The principle photon useful for the detection and imaging studies is gamma-2 with the mean energy of 140.5keV. The specific gamma ray constant for ^{99m}Tc is 0.8R/mCi-hr at 1cm (5.58μCi/kg/hr/MBq at 1cm). The use of 2.5mm thickness of lead can effectively attenuate the radiation emitted by a factor of 1000.

Principles of radiolabeling of compounds with ^{99m}Tc

The majority of ^{99m}Tc compounds employ the stannous chloride reduction method, which makes use of the fact that stannous chloride is one of the most powerful reducing agent. ^{99m}Tc obtained from the Mo / Tc generator is in chemical form of TcO_4^-, or pertechnetate. While the anion has an overall negative charge of -1, the oxidation number of technetium is +7. The chelating agents commonly used to prepare ^{99m}Tc products are also anions with an overall negative charge due to the presence of N, O and P atoms, each of which has 1 or more extra pairs of electrons. These negative charges repel each other so pertechnetate will not form chelates. A reducing agent is therefore required to convert the ^{99m}Tc into an electropositive cationic form capable of binding to chelating agents. ^{99m}Tc sulfur colloid

and ^{99m}Tc DMSA are the only two commercially available compounds that do not use the stannous reduction method. In the reaction, the stannous ion is the reducing agent, and therefore the substance oxidized, while pertechnetate is the oxidizing agent and therefore the substance reduced. Most soluble ^{99m}Tc compounds, excluding those containing a protein have octahedral structures and are said to be hexa coordinated since there are typical 6 binding sites available consisting of N, O, or P atoms.

8.2 Methods

8.2.1 Radiolabeling of Etoposide Formulations

Radiolabeling

The radiolabeling of ETPS, PLGA-ETP NP and Tf-PLGA-ETP NP was performed with ^{99m}Tc by direct labelling method [Richardson et. Al, 1977, Babbar A, Kashyap R, 1991]. Briefly, 1ml of ETPS, PLGA-ETP NP dispersion and Tf-PLGA-ETP NP dispersion (5mg/ml) each were mixed with 50µl of stannous chloride solution (5mg/ml). The pH was adjusted to 6.5 with 0.5M sodium bicarbonate solution. Further, the preparation was incubated with 100 µl of freshly eluted ^{99m}Tc-pertechnetate solution (2 mCi) for 30mins at room temperature. The quality control (percentage labeling efficiency and stability of the labelled complexes) was performed as described earlier. [Theobald, 1990]

The labeling efficiency of ^{99m}Tc-ETP/PLGA-ETP/Tf-PLGA-ETP was determined using ascending instant thin layer chromatography (ITLC) using silica gel (SG)-coated fibre glass sheets (Gelman Sciences Inc, Ann Arbor, MI). The ITLC was performed using acetone as the mobile phase. Approximately 2 to 3 µL of the radio-labeled complex was applied at a point 1 cm from one end of an ITLC-SG strip. The strip was eluted in acetone. The solvent front was allowed to reach 7-8 cm from the point of application. The strip was cut horizontally into 2 halves, and the radioactivity in each half was determined in a gamma ray counter (Gamma ray spectrometer, Captec-R, Capintec, USA). The free ^{99m}Tc-pertechnetate that moved with the solvent (R_f= 0.9) was determined.

The radiocolloids (reduced/hydrolyzed) technetium along with the labeled complex remained at the point of application. The amount of radiocolloids was determined using ITLC with pyridine: acetic acid: water (3:5:1.5 v/v) as mobile phase. The radiocolloids remained at the point of application, while both the free pertechnetate and the labeled complex moved away with the solvent front. The activity migrate using pyridine: acetic acid: water as a mixture was subtracted from that with the solvent front using acetone, the

net amount of ^{99m}Tc-ETPS, ^{99m}Tc-PLGA-ETP-NP, ^{99m}Tc-Tf-PLGA-ETP-NP, was calculated.

The radiolabelling was optimized for incubation time and the concentration of $SnCl_2.2H_2O$. The pH of the solution and the formulations was maintained at around 6.5. The influence of the incubation time and the concentration of $SnCl_2.2H_2O$ are given in table 8.1 and table 8.2 respectively.

Table 8.1: Influence of incubation time on the labeling efficiency of ^{99m}Tc-Etoposide formulations

Incubation time (mins)	% Radiolabeling		
	^{99m}Tc - **ETPS**	^{99m}Tc **-PLGA-ETP-NP**	^{99m}Tc **-Tf-PLGA-ETP-NP**
0	91.86 ±1.23	88.42 ± 1.02	90.26 ± 0.91
10	93.15 ±1.16	92.89 ± 1.43	93.78 ± 1.32
15	96.27 ±1.69	94.77 ± 1.31	96.24 ± 1.14
30	98.11 ±2.19	97.26 ± 2.26	97.64 ± 1.86
60	97.26 ±1.79	96.41 ± 1.46	97.16 ± 2.14

Table 8.2: Influence of the Amount of Stannous Chloride on the Labelling Efficiency of Etoposide formulations

$SnCl_2.2H_2O$ (µg) →	50	100	200	250	400
ETPS					
% labelling (mean ± SD)	87.21 ± 1.11	92.41 ± 0.96	94.17 ± 1.26	98.11 ± 0.83	95.62 ± 1.54
% colloids (mean ± SD)	0.72 ± 0.12	0.91±0.21	0.98±0.19	1.12 ±0.59	3.85 ± 0.92
% Free (mean ± SD)	12.07 ± 1.67	6.68 ±1.02	4.85 ± 1.23	0.77 ± 0.11	0.53 ± 0.25
PLGA-ETP-NP					
% labelling (mean ± SD)	68.25± 2.62	87.28 ± 1.08	92.77 ± 1.41	96.78 ± 0.96	95.36 ± 1.20
% colloids (mean ± SD)	0.89 ± 0.07	1.23 ± 0.34	1.29 ± 0.22	2.02 ± 0.62	3.32 ± 0.87
% Free (mean ± SD)	30.86 ± 2.12	11.49 ± 0.91	5.94 ± 1.31	1.2 ± 0.45	0.92 ± 0.15
Tf-PLGA-ETP-NP					
% labelling (mean ± SD)	57.81 ± 1.91	90.31 ± 2.31	92.07 ± 1.46	97.64 ± 1.21	93.33 ± 0.87
% colloids (mean ± SD)	0.68 ± 0.13	0.52 ± 0.25	1.12 ± 0.06	1.38 ± 0.61	4.98 ± 1.78
% Free (mean ± SD)	41.51 ± 1.94	9.17 ± 1.31	6.81± 1.62	1.19 + 0.94	1.69 ± 0.29

Each value is a mean of three experiments

In vitro Stability Study of Labeled Complex

The stability study of ^{99m}Tc-ETPS/PLGA-ETP-NP/Tf-PLGA-ETP-NP was determined in vitro using 0.9%w/v sodium chloride and rat serum by ascending thin layer chromatography. The complex (0.1 mL) was mixed with 1.9 mL of rat serum and incubated at 37°C. The samples at different time points upto 24hrs were subjected to ITLC using acetone solvent systems. The % labelling efficiency for etoposide solution and nanoparticles was determined. The results for stability in sodium chloride and rat serum are tabulated in table 8.3.

Table 8.3: Invitro stability of ^{99m}Tc-Etoposide formulations in 0.9%w/v sodium chloride and rat serum at 37°C

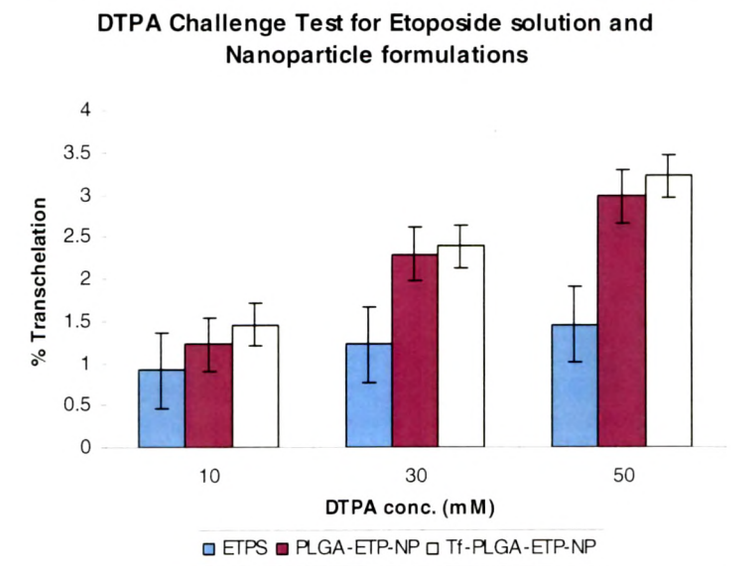
Time in hours	% Radiolabeling					
	In 0.90% Sodium Chloride			In Serum		
	ETPS	PLGA-ETP-NP	Tf-PLGA-ETP-NP	ETPS	PLGA-ETP-NP	Tf-PLGA-ETP-NP
0.5	98.02 ±0.82	96.61±0.62	97.52 ± 1.29	98.11 ±0.93	96.78±0.53	97.64±1.40
1	97.98 ±0.91	96.29 ± 1.24	97.06±1.32	98.02±0.86	94.56±1.81	97.55±1.69
2	97.44±0.64	95.67 ± 1.10	96.81 ±0.97	97.76±0.91	95.87±2.59	97.26±1.63
4	97.29 ± 1.12	95.19 ± 2.62	96.43 ± 1.20	97.82±1.14	94.92±0.97	96.84±1.83
6	96.92 ± 1.39	94..12 ± 2.19	96.16 ±2.37	97.56±0.79	94.16±1.38	96.59±1.20
24	95.18 ± 2.18	92.26 ± 1.46	92.47 ± 1.84	96.84±1.27	93.44±0.61	91.26±1.68

Transchelation challenge test using diethylene triamine penta acetic acid

To evaluate stability and bonding strength of the radio labeled solution/nanoparticles, one mL of the radio labeled formulation was challenged against various concentrations (10, 30 and 50 mM) of diethylene triamine penta acetic acid (Babbar A K et al., 2000). The mixtures were incubated for 4 hours at 37°C and the labeling efficiency was measured using ITLC-SG; acetone and PAW system as mobile phase. 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 was separated at migration value 0.90 (R_f = 0.90) while ^{99m}Tc-Etoposide

solution/nanoparticles remained at the point of application (R_f = 0). Effect of different molar concentration and percent transchelation is illustrated in Figure 8.1.

Figure 8.1: Effect of Variable molar concentration of DTPA on radiolabeled ^{99m}Tc complex of Etoposide formulations



8.2.2 Radiolabeling of Temozolomide Formulations

Radiolabeling

The radiolabeling of TMZS, PLGA-TMZ-NP and Tf-PLGA-TMZ-NP was performed with ^{99m}Tc by direct labelling method [Richardson et. Al, 1977, Babbar A, Kashyap R, 1991]. Briefly, 1ml of TMZS, PLGA-TMZ-NP and Tf-PLGA-TMZ-NP dispersion (prepared in 0.01NHCl, 10mg/ml) each were mixed with 50µl of stannous chloride solution (5mg/ml). The pH was adjusted to 5.0 with 0.5M sodium bicarbonate solution. Further, the preparation was incubated with 100 µl of freshly eluted ^{99m}Tc-pertechnetate solution (2 mCi) for 30mins at room temperature. The quality control (percentage labeling efficiency and stability of the labelled complexes) was performed as described earlier. [Theobald, 1990]

The labeling efficiency of ^{99m}Tc-TMZS/PLGA-TMZ-NP/Tf-PLGA-TMZ-NP was determined using ascending instant thin layer chromatography (ITLC) using silica gel (SG)-coated fibre glass sheets (Gelman Sciences Inc, Ann Arbor, MI). The ITLC was performed using acetone as the mobile phase. Approximately 2 to 3 µL of the radio-labeled complex was applied at a point 1 cm from one end of an ITLC-SG strip. The strip was eluted in acetone. The solvent front was allowed to reach 7-8 cm from the point of application. The strip was cut horizontally into 2 halves, and the radioactivity in each half was determined in a gamma ray counter (Gamma ray spectrometer, Captec-R, Capintec, USA). The free ^{99m}Tc-pertechnetate that moved with the solvent (R_f= 0.9) was determined.

The radiocolloids (reduced/hydrolyzed) technetium along with the labeled complex remained at the point of application. The amount of radiocolloids was determined using ITLC with pyridine: acetic acid: water (3:5:1.5 v/v) as mobile phase. The radiocolloids remained at the point of application, while both the free pertechnetate and the labeled complex moved away with the solvent front. The activity migrated using pyridine: acetic acid: water as a mixture was subtracted from that with the solvent front using acetone, the

net amount of ^{99m}Tc-TMZS, ^{99m}Tc-PLGA-TMZ-NP, ^{99m}Tc-Tf-PLGA-TMZ-NP, was calculated.

The radiolabeling was optimized for incubation time and the concentration of $SnCl_2.2H_2O$. The pH of the solution and the formulations was maintained at around 5.0. The influence of the incubation time and the concentration of $SnCl_2.2H_2O$ are given in table 8.4 and table 8.5 respectively.

Table 8.4: Influence of incubation time on the labeling efficiency of ^{99m}Tc-Temozolomide formulations

Incubation time (mins)	% Radiolabeling		
	^{99m}Tc - TMZS	^{99m}Tc -PLGA-TMZ-NP	^{99m}Tc -Tf-PLGA-TMZ-NP
0	90.17± 1.36	86.47 ± 2.11	91.5 ± 1.64
10	93.09 ± 2.23	91.84 ± 1.37	93.09 ± 1.27
15	95.77 ± 1.94	93.66 ± 1.42	94.25 ± 1.10
30	97.44 ± 1.59	96.42 ± 0.94	96.89 ± 1.47
60	96.89 ± 1.30	95.20 ± 1.29	96.27 ± 2.19

Table 8.5: Influence of the Amount of Stannous Chloride on the Labelling Efficiency of Temozolomide formulations

$SnCl_2.2H_2O$ (µg) →	50	100	200	250	400
TMZS					
% labelling (mean ± SD)	76.89 ± 0.78	82.41 ± 1.38	91.87 ± 1.31	97.23 ±0.83	96.46 ± 2.54
% colloids (mean ± SD)	0.56 ± 0.09	0.97±0.16	1.12±0.26	1.94 ±0.13	2.92 ± 0.21
% Free (mean ± SD)	22.55 ± 1.67	16.62 ±1.02	7.01 ± 1.23	0.83 ± 0.11	0.62 ± 0.09
PLGA-TMZ-NP					
% labelling (mean ± SD)	62.25±1.59	79.47 ± 0.89	94.28 ± 1.13	96.69 ± 0.91	94.39 ± 2.2
% colloids (mean ± SD)	1.23 ± 0.07	1.32 ± 0.34	1.92 ± 0.26	2.68 ± 0.28	5.39 ± 0.48
% Free (mean ± SD)	36.52 ±1.26	19.21 ± 1.43	3.8 ± 0.39	0.63 ± 0.17	0.22 ± 0.05
Tf-PLGA-TMZ-NP					
% labelling (mean ± SD)	69.76 ± 1.37	86.29 ± 1.66	94.83 ± 0.88	97.17 ± 1.94	93.89 ± 1.78
% colloids (mean ± SD)	0.76 ± 0.09	0.89 ± 0.11	1.15 ± 0.13	2.18 ± 0.21	5.69 ± 1.78
% Free (mean ± SD)	29.48 ± 2.1	12.82 ± 0.94	4.02± 0.44	0.65 + 0.08	0.42 ± 0.19

Each value is a mean of three experiments

In vitro Stability Study of Labeled Complex

The stability study of ^{99m}Tc-TMZS/PLGA-TMZ-NP/Tf-PLGA-TMZ-NP was determined in vitro using 0.9%w/v sodium chloride and rat serum by ascending thin layer chromatography. The complex (0.1 mL) was mixed with 1.9 mL of rat serum and incubated at 37°C. The samples at different time points upto 24hrs were subjected to ITLC using acetone solvent systems. The % labelling efficiency for etoposide solution and nanoparticles was determined. The results for stability in sodium chloride and rat serum are tabulated in table 8.6

Table 8.6: Invitro stability of ^{99m}Tc-Temozolomide formulations in 0.9%w/v sodium chloride and rat serum at 37°C

Time in hours	% Radiolabeling					
	In 0.90% Sodium Chloride			In Serum		
	TMZS	**PLGA-TMZ-NP**	**Tf-PLGA-TMZ-NP**	**TMZS**	**PLGA-TMZ-NP**	**Tf-PLGA-TMZ-NP**
0.5	97.17 ± 1.32	96.43 ± 1.43	97.04 ± 1.19	97.18 ± 1.12	96.44 ± 1.46	96.75 ± 2.41
1	96.85 ± 1.91	95.91 ± 1.62	96.44 ± 1.69	96.83 ± 1.34	96.27 ± 1.73	96.56 ± 1.52
2	96.53 ± 2.44	95.78 ± 1.98	95.75 ± 1.28	96.60 ± 1.59	95.78 ± 1.28	96.20 ± 1.64
4	96.15 ± 2.17	95.61 ± 1.77	95.12 ± 2.04	96.41 ± 1.74	95.37 ± 2.14	95.72 ± 1.19
6	95.86 ± 1.38	94.77 ± 2.18	94.92 ± 1.87	96.51 ± 1.41	94.69 ± 1.90	95.44 ± 1.73
24	94.89 ± 1.44	93.89 ±1.27	93.29 ± 1.43	95.84 ± 1.03	92.87 ± 1.68	93.29 ± 1.26

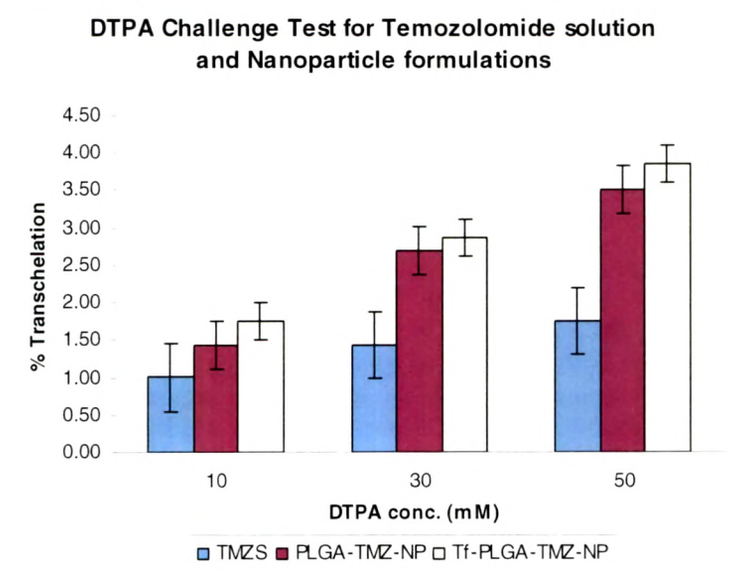
Each value is mean of three readings

Transchelation challenge test using diethylene triamine penta acetic acid

To evaluate stability and bonding strength of the radio labeled solution/nanoparticles, one mL of the radio labeled formulation was challenged against various concentrations (10, 30 and 50 mM) of diethylene triamine penta acetic acid (Babbar A K et al., 2000). The mixtures were incubated for 4 hours at 37°C and the labeling efficiency was measured using ITLC-SG; acetone and PAW system as mobile phase. 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 was separated at migration value 0.90 (R_f = 0.90) while ^{99m}Tc-Temozolomide

solution/nanoparticles remained at the point of application (R_f = 0). Effect of different molar concentration and percent transchelation is illustrated in Figure 8.2.

Figure 8.2: Effect of Variable molar concentration of DTPA on radiolabeled ^{99m}Tc complex of Temozolomide formulations



8.3 Discussions

ETPS, PLGA-ETP-NP and Tf-PLGA-ETP-NP were labelled with ^{99m}Tc with high labeling efficiency using direct labelling method. The quantity of stannous chloride to reduce ^{99m}Tc plays an important role in the labelling efficiency. Lower quantity of stannous chloride leads to low labelling efficiency where as higher amount of stannous chloride leads to formation of undesirable radiocolloids. The optimum quantity of stannous chloride for high labeling efficiency and low free and reduced/hydrolyzed ^{99m}Tc, was found to be 250μg for all preparations. The incubation time was optimized at 30mins. The pH for all the formulations was kept at around 6.5. The labeling efficiency and the stability of labeled complex were ascertained by ascending TLC using ITLC strips. The labelling efficiency for ETPS, PLGA-ETP-NP and Tf-PLGA-ETP-NP was found to be 98.11%, 96.78% and 97.64 % respectively. The invitro stability of radiolabelled preparations was checked in presence of rat serum and 0.9%w/v sodium chloride. Rat serum was selected to mimic the experiment in-vivo conditions related to serum proteins and physiological pH. The labelling efficiency of ^{99m}Tc labelled formulation at all the time points is found to be greater than 90%. Bonding strength of ^{99m}Tc-ETPS/PLGA-ETP-NP/Tf- PLGA-ETP-NP was also investigated by the DTPA challenging test, and the percent transchelation of the labeled complex was below 4%w/w at highest concentration tested (50mM). The results suggested high bonding strength and stability of ^{99m}Tc-ETPS/PLGA-ETP-NP/Tf- PLGA-ETP-NP. Thus these formulations were found suitable for biodistribution studies.

For Temozolomide, the optimum quantity of stannous chloride for high labeling efficiency and low free and reduced/hydrolyzed ^{99m}Tc, was found to be 250μg for all preparations. The incubation time was optimized at 30mins. The pH of all the formulations was kept at around 5. The labelling efficiency for TMZS, PLGA-TMZ-NP and Tf-PLGA-TMZ-NP was found to be 97.23%, 96.69% and 97.17 % respectively. The radiolabelled complex show high stability in rat serum and 0.9%w/v sodium chloride with Radiolabeling efficiencies measured, greater than 90%. The DTPA challenging test, suggest high stability of the radiolabelled complex, with percent transchelation below 4%w/w at 50mM.

Direct radiolabeling was found to be useful tool to study biodistribution. Radiolabeling of nanoparticles and solutions of Etoposide and Temozolomide were successfully performed and the results indicated good stability and bonding strength of the radiolabeled complex. Hence, these formulations were found stable and suitable to study biodistribution and to study gamma scintigraphy imaging of these formulations on animals.

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