Chapter 5

Radiolabeling techniques

5.1 RADIOLABELING OF PHARMACEUTICAL SUBSTANCES AND ITS APPLICATIONS

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 prefereable 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 radiolabelled 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. Technicium 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 (Martin MJ., 1976). 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 technicium 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.

5.2 RADIOLABELING OF OLANZAPINE, OLANZAPINE NANOSUSPENSION AND OLANZAPINE LOADED SOLID LIPID NANOPARTICLE FORMULATIONS

Methods

Radiolabeling

Olanzapine, Olanzapine nanosuspension and Olanzapine loaded solid lipid nanoparticles were radiolabelled with ^{99m}Tc by reduction with stannous chloride similar to the methods reported earlier (Richardson VJ et al., 1977; Theobald AE., 1990). Briefly, the optimized quantity of stannous chloride (exact quantity of stannous chloride required shown in table 5.1) was added to the respective formulation. OL was radiolabelled as follows: Sufficient olanzapine (OL) was dissolved in 0.01N hydrochloric acid to get a 1mg/mL solution of OL in 0.01N hydrochloric acid. Then the pH was adjusted to 5.5-6.0 using0.5M sodium bicarbonate. Then ^{99m}Tc (2 mCi) was added to the above solution and incubated at room temperature for 15 minutes. In case of the OL loaded nanoparticle formulations, the lyophilized powder equivalent to 1mg OL was accurately weighed (~ 40mg) and dissolved in 1mL of double distilled water to get a 1mg/mL solution of OL. The pH was then adjusted to 5.5 to 6.0 with 0.5 M sodium bicarbonate. Then ^{99m}Tc (2 mCi) was added to the above solution and incubated at room temperature for 15 minutes.

Quality control was performed as per the method reported earlier (Theobald AE., 1990) The labeling efficiency of OL and the various nanoparticle formulation was determined by instant ascending thin layer chromatography (TLC) using silica gel-coated fiber sheets (Gelman Sciences Inc, Ann Arbor, MI). The instant thin layer chromatography (ITLC) strip was spotted with 2 to 3 µL of the labeled complex at 1 cm up from the bottom and developed using acetone as the mobile phase. The solvent front was allowed to reach up to a height of ~8 cm from the origin. The strip was cut into 2 halves, and the radioactivity in each half was determined by a well-type gamma ray spectrometer (type GRS23C, Electronics Corporation of India Ltd, Hyderabad, India). The free pertechnetate having an Rf value in-between 0.9-1.0 migrates to the top portion of the ITLC strip, leaving the reduced/hydrolyzed ^{99m}Tc along with the labeled complex at the bottom. Incorporation of excess of stannous chloride for reduction of ^{99m}Tc may lead to the formation of radio colloids, which is undesirable.

The colloid formation was determined in pyridine: acetic acid: water (3:5:1.5). The radio colloids remained at the bottom of the strip, while both the free pertechnetate and the labeled

complex migrated with the solvent front. By subtracting the migrated activity with the solvent front using acetone from that using pyridine: acetic acid: water mixture, the net amount of ^{99m}Tc-OL, ^{99m}Tc-OL nanosuspension and ^{99m}Tc-OL loaded SLN was calculated.

Stability of the 99mTc labeled complexes

Determination of invitro serum stability of the radiolabelled complex is an important parameter to be determined because the serum proteins can chelate and bind with the ^{99m}Tc, disturbing the stability of the radiolabelled complex. In vitro serum stability can also throw light on the stability of the radiolabelled complex in biological environment once injected into the body. Stability of the ^{99m}Tc-labeled complexes of OL and the various OL formulations was determined in vitro in human serum by ascending TLC technique. The labeled complex (0.1 mL) was incubated with freshly collected human serum (0.4 mL) at 37°C. The samples were withdrawn at regular intervals up to 24 hours and analyzed in gamma ray spectrometer.

DTPA and Cysteine Challenge tests

These studies were performed in order to check the strength of binding of ^{99m}Tc with the compounds. The studies were performed as previously reported (Mishra A.K., 2002). In brief, fresh solutions of DTPA and cysteine (10, 30, and 50 mM) were prepared in 0.9% saline. Five hundred microliters of the labeled preparation was treated with different concentrations of DTPA and cysteine separately and incubated for 1 hour at 37°C. Five hundred microliters of 0.9% saline served as control. The effect of DTPA and cysteine on the labeling efficiency of complexes was measured by ITLC-silica gel strips using acetone as mobile phase. In this system ^{99m}Tc-OL, ^{99m}Tc-OL nanosuspension and ^{99m}Tc-OL loaded SLN complexes remain at the origin (Rf = 0.0), while pertechnetate (Rf = 0.9-1.0) and all known chemical forms of ^{99m}Tc-DTPA and ^{99m}Tc-cysteine complexes migrate (Rf = 0.7-1.0). After developing, each paper was cut into 2 halves and each half was counted for radioactivity in gamma ray spectrometer.

5.3 RESULTS AND DISCUSSION

Radiolabeling

High labeling efficiency was achieved for the OL and its various nanoparticle formulations. The amount of stannous chloride added played a vital role in the labeling process. The influence of stannous chloride on the labeling efficiency and the formation of colloids are

shown in table 5.3a and 5.3b. After initial experiments the optimum pH required for maximum radiolabeling was found to be 5.5 to 6.0. Lower amounts of stannous chloride lead to poor labeling efficiencies because of insufficient reduction of pertechnetate to its lower valence state. Higher concentrations of stannous chloride lead to formation of higher amount of undesirable radiocolloids. The labeling efficiency and stability of the labeled complex were determined by thin layer chromatography.

Stability of the ^{99m}Tc labeled complexes

In vitro serum stability of ^{99m}Tc-labeled complexes of OL and the other OL nanoparticle formulations was determined up to 24 hours. The results obtained are tabulated in table 5.2. The data demonstrated that the labeled complexes remained stable in serum upto a time period of 24 hours. The serum stability of the labeled complexes indicates their use as potential markers for blood clearance and biodistribution studies.

DTPA and Cysteine Challenge tests

In vitro stability of the radiolabeled complexes was determined by DTPA and cysteine challenge studies. Challenge studies demonstrated that the labeling efficiency of the complexes did not alter much in the presence of DTPA (Figure 5.1) and cysteine (Figure 5.2). Even at 50 mM concentration of DTPA and cysteine, the transchelation was found to be less than 4%, indicating the stability of the radiolabeled complexes. These data further potentiate the usefulness of these biomarkers as possible biomarkers for blood clearance and biodistribution studies.

Formulation	Amount of Stannous chloride	pH	Incubation time
Olanzapine Solution	100µg	5.5 to 6.0	15 minutes
Poloxamer stabilized Olanzapine nanosuspension (POL NS)	100µg	5.5 to 6.0	15 minutes
TPGS stabilized Olanzapine nanosuspension (TOL NS)	100µg	5.5 to 6.0	15 minutes
Olanzapine in 3% Gelucire Solution (GOL Soln.)	150µg	5.5 to 6.0	15 minutes
Olanzapine in 5% Transcutol Solution (TROL Soln.)	150µg	5.5 to 6.0	15 minutes
Gelucire® stabilized Olanzapine loaded solid lipid nanoparticles (GOL SLN)	150µg	5.5 to 6.0	15 minutes
Transcutol® stabilized Olanzapine loaded solid lipid nanoparticles (TROL SLN)	200µg	5.5 to 6.0	15 minutes
Poloxamer 188 stabilized Olanzapine loaded solid lipid nanoparticles (POL188 SLN)	200µg	5.5 to 6.0	15 minutes

Table 5.1: Amount of stannous chloride, pH to be maintained and the incubation time required for the radiolabeling of Olanzapine and the various Olanzapine nanoparticle formulations.

Time (h)	% Radiolabelling							
	OL Soln	POL NS	TOL NS	GOL Soln	TROL Soln	GOL SLN	TROL SLN	POL188 SLN
Initial	99.23	98.92	99.53	99.41	99.61	99.14	99.07	98.85
0.25	99.19	98.88	99.50	99.39	99.60	99.10	99.03	98.76
0.5	99.18	98.80	99.49	99.36	99.57	99.01	98.94	98.60
1	99.09	98.77	99.46	99.28	99.48	98.90	98.78	98.42
2	98.89	98.62	99.32	99.21	99.40	98.74	98.64	98.06
4	98.63	98.38	99.25	99.10	99.12	98.59	98.41	97.73
8	98.22	98.09	99.03	98.85	98.83	98.06	98.23	97.38
24	97.53	97.04	98.45	98.14	97.95	97.67	97.16	96.67

Table 5.2: Stability data of the various ^{99m}Tc - radiolabelled complexes in human serum

Amount of Stannous Chloride added	% Radiobelling		
	Olanzapine Solution		
	% L	% C	% F
50µg	76.4	0.3	23.3
100 µg	99.2	0.7	0.1
125 µg	96.9	2.5	0.6
150 µg	94.4	4.5	1.1

Table 5.3a: Influence of amount of reducing agent stannous chloride on the percent radiolabeling of Olanzapine

Amount of Stannous Chloride added	% Radiolabelling																				
	POL NS			TOL NS			GOL Soln.			TROL Soln.			POL188 SLN			GOL SLN			TROL SLN		
	% L	% C	% F	% L	% C	% F	% L	% C	% F	% L	% C	% F	% L	% C	% F	% L	% C	% F	% L	% C	% F
50µg	81.6	0.7	17.7	77.6	0.6	21.8	82.3	0.6	17.1	80.6	0.4	19	84.7	0.3	15	76.7	0.4	19.7	84.2	0.6	15.2
100 µg	98.9	0.4	0.7	99.5	0.4	0.1	86.1	0.2	13.7	89.6	0.4	10	90.1	0.2	9.7	85.4	0.6	13.3	87.4	0.4	12.2
125 µg	97.0	2.2	0.8	96.5	3.0	0.5	90.5	0.9	8.6	96.7	0.3	3	93.3	0.6	6.1	91.0	0.5	9.8	90.5	0.2	9.3
150 µg	93.8	4.2	2.0	92.7	4.9	2.4	99.4	0.5	0.1	99.6	0.3	0.1	99.1	0.4	0.5	95.3	0.6	1.6	96.0	0.2	3.8
200 µg	-	-	-	-	-	-	96.0	3.8	0.2	97.5	2.3	0.2	96.8	0.6	0.6	99.0	0.5	0.5	98.9	0.4	0.7
250 µg	-	-	-	-	-	-	94.2	5.2	0.6	94.0	5.1	0.9	95.1	3.9	1.0	97.7	1.7	0.4	96.2	2.9	0.9

% L: Percentage radiolabelled; % C Percentage: radiocolloids; % F: Percentage free pertechnetate.

Table 5.3b: Influence of amount of reducing agent stannous chloride on the percent radiolabeling of the various Olanzapine formulations

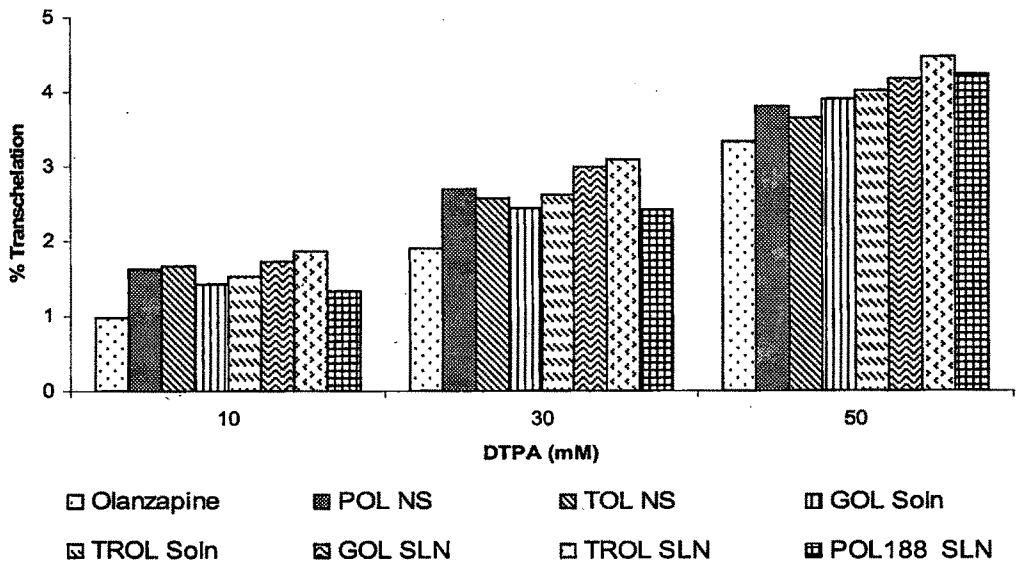

Figure 5.1: Determination of in vitro stability of the ^{99m}Tc-labelled complexes of olanzapine and the different olanzapine nanoparticles by DTPA challenge test

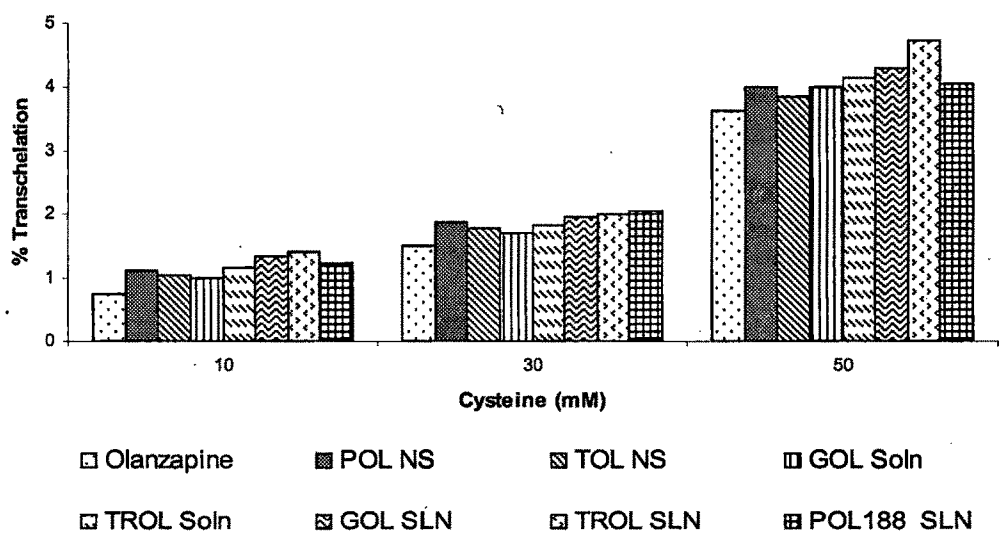

Figure 5.2: Determination of in vitro stability of the ^{99m}Tc-labelled complexes of olanzapine and the different olanzapine nanoparticles by Cysteine challenge test

5.4 REFERENCES

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