Chapter 6

RADIOLABELLING OF CELECOXIB AND ITS FORMULATIONS

6.1 Introduction

Radiolabelling has proven to be quite useful for following the fate of the injected formulation in-vivo and also as diagnostic agent. The radiolabelling is a simple and less time consuming technique to study the bio-distribution of the injected drug in-vivo compared to the methods like high performance liquid chromatography and other analytical methods. In the present investigation, the bio-distribution of gelatin microspheres, chitosan microspheres, bovine serum albumin microspheres and solid lipid nanoparticles containing celecoxib after intra-articular injection was compared with that of the celecoxib solution. Also, the biodistribution of the celecoxib loaded albumin microspheres and solid lipid nanoparticles after intra-venous injection in rats was studied. The drug, celecoxib and its formulations were radiolabeled with technetium and the extra-articular distribution of the drug and its formulations after intra-articular injection was studied by measuring the radioactivity present in the joints and the other organs at specific time intervals.

^{99m}Tc is the best candidate for imaging studies due to its short life, pure photon emitter and suitable energy. 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 used is its extremely favorable physical and radiation characteristics. The 6 hour physical half life and the little amount of electron emission permit the administration of mill curie 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 ^{99m}Mo- ^{99m}Tc generators.

6.2 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 ^{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 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 +7and + 4 states are the most stable and are represented in oxides, sulphides, halides and pertechnetate. 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 oxidized to +4 state and finally to the +7 state.

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

The chemical form of 99m-Technetium available from the Molybdenum generator is sodium pertechnetate (^{99m}Tc-$NaTcO_4$). The pertechnetate ion, $^{99m}TcO_4^-$, having the oxidation state + 7for ^{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-labelling of many compounds, prior reduction of ^{99m}Tc from +7 state to a lower oxidation state is required. Various reducing agents that have been used are stannous chloride, stannous tartarate, concentrated HCl, Sodium borohydride ($NaBH_4$), dithionate 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.

6.4 Labelling with reduced Technetium

The reduced ^{99m}Tc species are chemically reactive and combine with a wide variety of compounds, which usually donate 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 labelling with technetium.

6.5 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 colloid. These colloids bind to reduced ^{99m}Tc and thus compromise labelling yield. To prevent this colloid formation, an acid is added to prevent the hydrolysis of Sn^{2+} before the reduction of technetium.

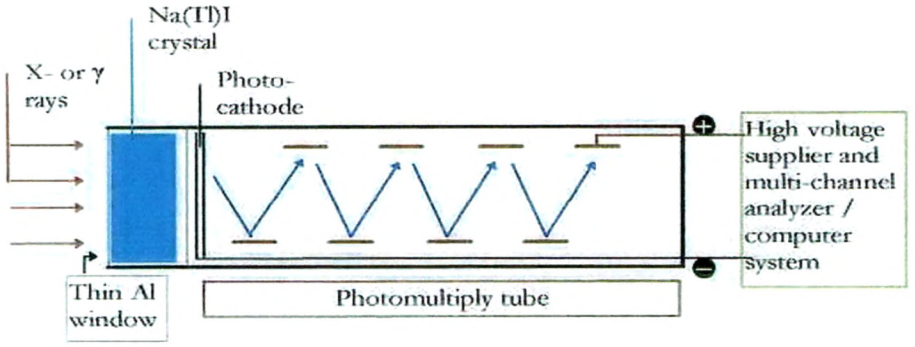
6.6 Principle of working of gamma scintillation counter and gamma camera

Scintillation counters are commonly used for X-rays and gamma rays. The name suggests that the working principle for scintillation counters is based on light emission.

The gamma scintillation counter consists of a sodium Iodide crystal which is enclosed in a stainless steel casing. It also contains 0.5 mole percent of thallium iodide as an activator. This is optically coupled to photomultiplier tube which is connected to a pre-amplifier. The pre-amplifier is connected to a linear amplifier, pulse height analyzer or single channel analyzer which amplifies the pulses linearly. The amplified pulse can be then recorded using a scaler. The output pulses from a scintillation counter are

proportional to the energy of the radiation. Photons striking a sodium iodide (NaI) crystal, which contains 0.5 mole percent of thallium iodide (TlI) as an activator, cause the emission of a short flash of light in the wavelength range of 3300-5000 A (in the ultraviolet region). The light flashes are detected by a photomultiplier tube, which gives a pulse corresponding to the light intensity. These pulses are measured by a multi-channel counter. In case of gamma camera, the scaler is substituted by a camera which can non-invasively detect the presence of the radioactivity in the body.

The Key Components of a Typical Scintillation Counter



6.7 Experimental

6.7.1 Materials

Diethylene triamine penta acetic acid (DTPA) and stannous chloride dehydrate (SnCl2. 2H2O) 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.

6.7.2 Radiolabelling of celecoxib and its formulations

The radiolabelling of celecoxib and its microsphere formulations with reduced ^{99m}Tc were carried out as per the procedure given below:

Celecoxib and the celecoxib-loaded formulations were labeled with ^{99m}Tc by direct labelling method as described earlier (Richardson et al, 1977). Briefly, 1mL of ^{99m}Tc (2mCi/ml) was mixed with 60 µl of stannous chloride solution in 10% acetic acid (1mg/ml) for celecoxib solution, solid lipid nanoparticles and albumin microspheres. For labelling of chitosan and gelatin microspheres 100µl of stannous chloride solution was used. The pH was adjusted to 6.5-7.0 using 0.5M sodium bicarbonate solution. To this mixture, 1ml of the celecoxib solution (2mg/ml) or the microsphere suspension (30–40mg/ml) or solid lipid nanoparticles suspension (1 ml) was added and incubated for 10 min at room temperature. The quality control was performed as per the method described earlier (Theobald, 1990).

6.7.3 Labelling efficiency

The radiochemical purity of ^{99m}Tc with celecoxib or the 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. Around 2–3µl of the radiolabeled complex was applied at a point 1 cm from the 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 spectrometer, Type GRS23C; Electronics Corporation of India Ltd, Mumbai). The free pertechnetate, which moved with the solvent (Rf =0.9), was determined. The reduced/hydrolysed (R/H) technetium, along with the labelled complex, remained at the point of application. The amount of

R/H 99mTc (radiocolloids) was determined using pyridine–acetic acid–water (3:5:1.5 v/v) as mobile phase. The radiocolloids remained at the point of application while 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 radiolabeled celecoxib, as well as microspheres, was calculated.

6.7.4 Stability study of ^{99m}Tc–celecoxib/formulations complex

The stability of the radiolabeled complex was determined in-vitro using 0.9% sodium chloride and serum by ascending thin-layer chromatography. The radiolabeled complex (0.1 ml) was mixed with 1.9ml of normal saline or human serum and incubated at 37°C. ITLC was performed at different time intervals to determine the stability of the complex.

6.7.5 Transchelation with DTPA

To check the stability and strength of the binding of 99mTc with celecoxib or celecoxib-loaded microspheres, 0.5ml of 1.0mM solutions of DTPA in saline were taken in separate 5 ml vials. To these vials, 0.1mL of the radiolabeled celecoxib/formulations was added. After brief mixing, each vial was incubated for 1 h at 37°C. As a control, 0.5mL of saline was mixed with 0.1mL of the labelled preparation and incubated for 1 h at 37°C. The effect of DTPA on the labelling efficiency was determined using ITLC-SG using normal saline as the mobile phase, which allowed the separation of free pertechnetate and DTPA complex (Rf=0.8–1) from the ^{99m}Tc–celecoxib/microsphere complex, which remained at the point of application (Rf=0).

6.7.6 Optimization of radiolabelling of celecoxib and its formulations

The effect of stannous chloride concentration on the labelling efficiency was studied to obtain optimum concentration needed for maximum labelling as shown in table 6.1 and table 6.2. The effect of pH on the labelling efficiency of ^{99m}Tc– celecoxib/formulations was studied by determining the labelling efficiency, keeping the other variables constant and changing the pH. The results are shown in table 6.3 and 6.4 and figure 6.1 and 6.2. To study the effect of incubation time on the efficiency, the radiolabelled complexes were incubated for various time periods keeping the other variables constant. The results are shown in table 6.5 and 6.6 and figures 6.3 and 6.4. The in-vitro stability studies of the labelled complexes were carried out in saline and in serum and the results are shown in table 6.7 and 6.8.

6.7.7 Data analysis

The data was analyzed using a multiple comparison Kruskal-Wallis test followed by post hoc Dunn's test at a significance level of $p<0.05$ and $p<0.01$.

Table 6.1: Effect of stannous chloride concentration on the Labelling efficiency of CS, CMS and AMS

$SnCl_2.2H_2O$ µg	CS			CMS			AMS		
	% bound	% colloids	% free	% bound	% colloids	% free	% bound	% colloids	% free
30	86.8±1.95	1.44±0.07	11.76±1.01	70.82±1.83	3.84±0.41	25.34±0.98	85.91±0.76	5.05±1.74	9.04±1.66
60	98.65±1.01	0.88±0.17	0.47±0.13	75.64±1.51	5.67±0.32	18.63±1.12	95.85±0.46	3.46±1.11	0.69±0.38
100	95.51±1.09	2.70±0.37	1.79±0.14	94.60±0.74	3.55±0.87	1.85±0.72	91.87±1.29	5.64±1.16	2.49±1.42
150	88.65±1.75	7.84±1.21	3.51±0.26	89.11±1.85	10.42±1.10	0.47±0.18	87.75±1.11	11.52±1.08	0.73±0.79

The results are expressed as mean ± S.D (n=3)

Table 6.2: Effect of stannous chloride concentration on the Labelling efficiency of SLN and GMS

$SnCl_2.2H_2O$ µg	SLN			GMS		
	% bound	% colloids	% free	% bound	% colloids	% free
30	88.63±1.05	0.90±0.15	10.47±1.20	85.42±0.92	1.01±0.26	13.57±0.72
60	98.79±0.47	0.58±0.25	0.63±0.38	91.61±0.90	1.86±0.30	6.53±0.98
100	94.20±0.92	5.38±0.67	0.42±0.25	94.84±1.10	2.78±0.50	2.38±1.44
150	85.77±0.77	12.85±1.34	1.38±1.02	90.25±1.34	1.55±0.44	8.20±1.67

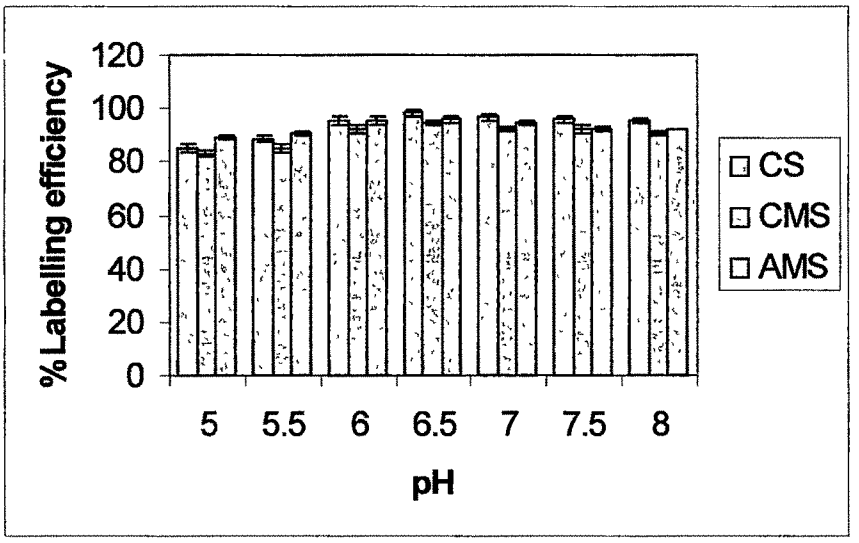
The results are expressed as mean ± S.D (n=3)

Table 6.3: Effect of pH on the labelling efficiency of CS, CMS and AMS

pH	% labelling efficiency		
	CS	CMS	AMS
5.0	85±1.60	82.76±1.26	88.91±0.78
5.5	88.34±1.14	85.14±1.66	90.63±1.15
6.0	95.68±1.56	92.17±1.32	95.13±1.69
6.5	98.17±0.86	94.55±0.87	95.85±0.89
7.0	96.72±1.07	92.25±0.85	94.4±1.00
7.5	95.81±1.39	92.12±1.36	92.06±0.80
8.0	95.63±0.69	90.63±1.09	92.17±0.30

All the values are expressed as mean ± S.D(n=3)

Figure 6.1: Effect of pH on the labelling efficiency of CS, CMS and AMS



The error bars in the chart represents the standard deviation values (n=3)

Table 6.4: Effect of pH on the labelling efficiency of SLN and GMS

pH	% labelling efficiency	
	SLN	GMS
5.0	82.61±1.66	86.35±1.35
5.5	87.24±1.19	88.73±2.75
6.0	93.06±2.43	90.38±1.57
6.5	95.63±1.06	94.55±1.24
7.0	99.18±0.71	90.8±1.71
7.5	94.82±2.20	85.31±0.49
8.0	90.78±1.79	86.3±1.75

Figure 6.2: Effect of pH on the labelling efficiency of SLN and GMS

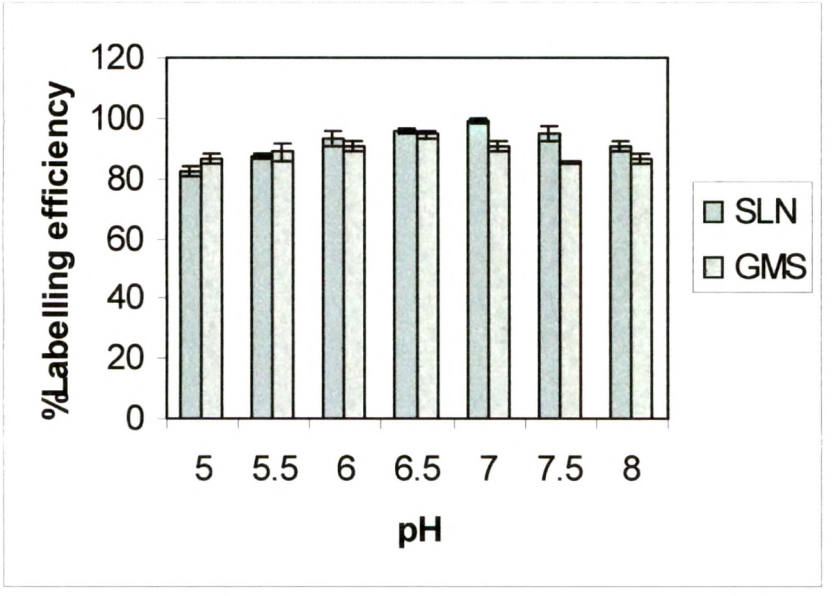


Table 6.5: Effect of Incubation time on the labelling efficiency of CS, CMS and AMS

Incubation time(mins)	% Radiolabelled		
	CS	CMS	AMS
	92.45±1.22	92.73±1.2	93.61±0.97
5	95.63±1.04	93.45±1.24	94.19±0.6
10	98.65±1.01	94.60±0.74	95.85±0.87
20	98.26±1.72	94.12±1.55	95.32±0.43
30	97.84±0.41	93.81±1.12	95.18±1.12

All the values are expressed as mean ± S.D (n=3)

Figure 6.3: Effect of incubation time on the labelling efficiency of CS, CMS and AMS

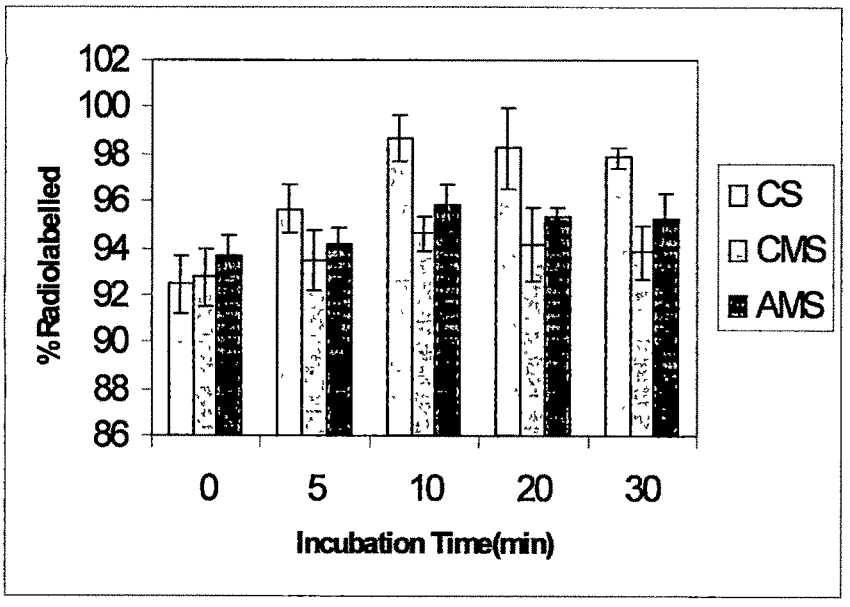


Table 6.6: Effect of Incubation time on the labelling efficiency of SLN and GMS

Incubation time (mins)	% Radiolabelled	
	SLN	GMS
0	95.77±1.36	93.55±1.25
5	98.79±0.19	93.71±0.50
10	98.04±0.38	94.84±0.71
20	97.57±1.19	94.66±0.81
30	97.38±0.78	93.74±2.25

Figure 6.4: Effect of Incubation time on the labelling efficiency of SLN and GMS

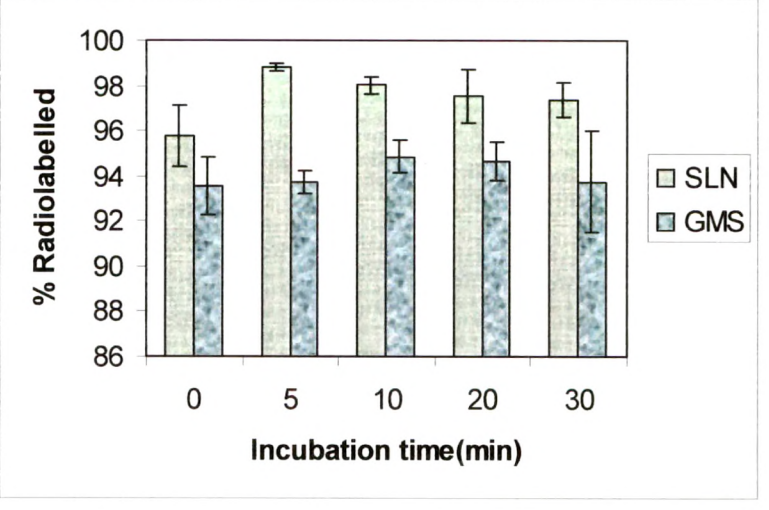


Table 6.7: Stability of the ^{99m}Tc-Celecoxib (CS) and ^{99m}Tc-formulations (CMS, AMS, SLN and GMS) in physiological saline at 37°C

Time in hours	% Radiolabelled				
	CS	CMS	AMS	SLN	GMS
0	98.65±1.01	94.60±0.74	95.85±0.99	98.79±1.01	94.84±1.07
0.25	98.40±0.58	94.57±0.82	95.61±1.25	98.63±1.17	94.75±0.69
0.5	98.32±1.46	94.41±1.33	95.35±2.17	98.37±0.68	94.6±1.33
1	97.81±1.57	94.30±1.03	95.28±1.38	97.66±0.76	93.83±1.21
2	97.46±0.76	93.66±0.69	94.91±1.90	97.1±0.36	93.75±1.35
4	97.20±1.14	93.14±1.22	94.56±1.10	96.8±1.39	92.3±0.57
24	96.65±0.93	92.72±1.23	93.82±1.06	93.53±0.90	90.68±1.15

The samples were subjected to ITLC (SG) with 100% acetone as the mobile phase. Data are expressed as percentage of the total radioactivity in sample. All the values are expressed as mean ± S.D

Table 6.8: Stability of the ^{99m}Tc-Celecoxib (CS) and ^{99m}Tc-Formulations (CMS, AMS, SLN, GMS) in serum in-vitro at 37°C

Time in hours	% Radiolabeled				
	CS	CMS	AMS	SLN	GMS
0	98.48±1.43	94.34±0.45	95.72±0.99	99.05±0.28	94.61±0.93
0.25	98.30±1.17	94.30±1.25	95.18±1.99	98.84±0.43	94.5±0.89
0.5	97.56±1.18	94.13±1.27	94.63±1.08	98.61±0.97	94.28±1.04
1	97.14±1.33	93.24±0.91	94.17±1.82	98.43±1.21	93.58±0.45
2	96.82±0.82	93.75±1.12	94.85±1.25	97.73±0.77	93.22±0.39
4	96.14±1.42	92.46±1.44	93.71±1.18	97.48±0.61	92.62±0.21
24	94.50±0.80	92.13±1.00	92.66±2.37	94.5±1.15	90.76±1.10

The samples were subjected to ITLC (SG) with 100% acetone as the mobile phase. Data are expressed as percentage of the total radioactivity in sample. All the values are expressed as mean ± S.D

Table 6.9: Transchelation of the radiolabelled complexes with DTPA

Complex	% Radiolabeled	
	Control	DTPA
CS-^{99m}Tc complex	97.56±1.21	95.35±1.36
CMS-^{99m}Tc complex	94.33±1.25	91.46±0.83
AMS-^{99m}Tc complex	95.67±0.75	91.46±0.81
SLN-^{99m}Tc complex	98.64±1.40	92.75±0.63
GMS-^{99m}Tc complex	94.82±0.82	90.71±1.35

All the values are expressed as mean±S.D (n=3)

6.8 RESULTS AND DISCUSSION

Radiolabelling has proven to be quite useful in following the fate of injected microspheres in-vivo and also as diagnostic agent. ^{99m}Tc is the best candidate for radiolabelling due to it having a short half-life, being a pure photon emitter and having suitable energy. Chemically, $TcO4^-$ is a rather non-reactive species and does not label any compound by direct addition. In 99mTc-of many compounds, prior reduction of ^{99m}Tc from +7 state to a lower oxidation state is required. Various reducing agents that have been used include stannous chloride, stannous citrate, sodium borohydride etc. Among these, stannous chloride is the most commonly used reducing agent in acidic medium in most preparations of ^{99m}Tc-labelled compounds. The reduced ^{99m}Tc species are chemically reactive and combine with a wide variety of compounds having $-COO^-$, $-OH^-$, $-NH_2$ and –SH by forming co-ordinate covalent bonds. Since celecoxib, chitosan, gelatin and albumin all contains a free NH_2 group, with ^{99m}Tc was possible. Celecoxib and its microspheres were labelled with high efficiency by the direct technique using reduced ^{99m}Tc. Data on radiochemical purity and stability of the labelled complex was obtained by ascending chromatography by

eluting with 0.9%saline or 100%acetone. The radiochemical impurities were free $TcO4^-$ and reduced/ hydrolysed ^{99m}Tc (radio colloids) in the ^{99m}Tc-labelled complexes. The pertechnetate used for the study was first reduced to its lower valency state using stannous chloride dihydrate and then the pH was adjusted to 6.5 before mixing with the celecoxib solution/ microsphere suspension. The radiolabelling was optimized by taking three factors into consideration (pH of the complex, stannous chloride dihydrate concentration and incubation time). The influence of the $SnCl_2.2H_2O$ concentration on the labelling efficiency was studied by varying its concentration (30–150 μg) and keeping the pH constant (6.5). The results are shown in Tables 6.1 and 6.2. The labelling efficiency of celecoxib increased from 86.8 to 98.65% when the stannous chloride concentration was increased from 30 to 60 μg. Further increase in the concentration of stannous chloride led to a decrease in the labelling efficiency. There was a significant difference ($P<0.05$) in the labelling efficiency of celecoxib when different concentrations of $SnCl_2.2H_2O$ were used. With chitosan microspheres and gelatin microspheres, an increase in the concentration of stannous chloride from 30 to 100 μg led to a significant increase ($P<0.05$) in the efficiency from 70.82 to 94.60% and 84.82 to 94.82% respectively. In case of solid lipid nanoparticles and albumin microspheres, the optimum concentration of stannous chloride for maximum efficiency was found to be 60μg. Further increase in the stannous chloride concentration led to a decrease in the efficiency. Also, in both the drug and the microspheres, when a lower concentration of stannous chloride was used, the remaining activity was as free $TcO4^-$ while at higher concentrations of stannous chloride, amount of radiocolloids increased.

Tables 6.3 and 6.4 and figures 6.1 and 6.2 depicts the effect of pH on the labelling efficiency. As the pH increased from 5 to 6.5, the labelling efficiency also increased

from 85% to 98.17% for celecoxib solution and from 82.76% to 94.55%for chitosan microspheres. For Albumin microspheres and gelatin microspheres, an increase in the pH from 5.0 to 6.5 led to an increase in the labelling efficiency from 88.91% to 95.85% and from 86.35% to 94.55% respectively. In case of solid lipid nanoparticles, an increase in the pH from 5.0 to 7.0 led to an increase in the labelling efficiency from 82.61% to 99.18%. With further increase in the pH, there was a decrease in the efficiency. This indicates that there was a significant effect ($P<0.05$) of pH on the efficiency of celecoxib and celecoxib-loaded formulations. Dunn's post-hoc test showed that this effect was highly significant between pH 5.5 and 7.0.

The incubation time required for high labelling efficiency was found to be 10 min for celecoxib, its chitosan, albumin and gelatin microspheres (Table 6.5 and 6.6). For, solid lipid nanoparticles, the optimum incubation time was found to be 5 minutes (Table 6.6). Further increase in the incubation time did not increase the efficiency considerably. There was a significant difference ($P<0.05$) in the efficiency of celecoxib and the formulations when incubated for different time intervals.

The stability of the labelled celecoxib and celecoxib loaded formulations was studied in saline and serum at 37°C (Table 6.7 and 6.8). Even after 24 h of incubation, there was only a 5–6% decrease in the labelled compound, indicating the high stability of the labelled complex. There was no significant difference ($P>0.05$) in the percentage of radiolabelled celecoxib or formulations at different time intervals either in saline or in serum. Further, the stability was confirmed by challenging the labelled complex with DTPA solution. As shown in Table 6.9, there was a 4–5% decrease in the labelled complex in the presence of DTPA. This

decrease was only marginal and was due to the high affinity of the DTPA for ^{99m}Tc. The Mann–Whitney U-test showed that there was no significant difference ($P>0.05$) in the percentage of either radiolabeled celecoxib or microspheres between the control and DTPA group.

6.9 References

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Theobald AE. Chapter 7 in: Textbook of radiopharmacy: theory and practice. 1990, Sampson, CB, ed., Gordon & Breach, New York, pp 127–129.