Chapter 8

IN-VIVO STUDIES-
γ-SCINTIGRAPHY

8.1 Introduction

This study involves the use of γ-scintigraphy for assessment of arthritis. Evaluation of arthritis was done by injecting intra-venously ^{99m}Tc-glutathione which preferentially accumulates in the inflamed region of the arthritic joint. Imaging was then done using a gamma camera, which clearly shows the arthritic lesions. A comparison was made between the duration of anti-inflammatory effect of celecoxib and its different formulations by taking the scintigraphic images of the rats at different time intervals.

8.2 Principle of Gamma scintigraphy

Gamma scintigraphy is a technique whereby the transit of a dosage form through its intended site of delivery can be non-invasively imaged *in vivo* via the judicious introduction of an appropriate short lived gamma emitting radioisotope. It is also used for diagnosis of certain diseased conditions whereby a radiolabelled molecule acts as a marker for the disease. Gamma scintigraphy relies on the administration of a radiopharmaceutical which is a gamma ray emitter with a relatively short half life. Bound to the radioisotope is a molecule which is taken up selectively by specific tissues. The radiolabelled molecule becomes concentrated in areas of diseased site. This accumulation is then imaged using a 'gamma camera' - a form of a scintillation counter, combined with a computer to process the image. Research continues on the applications of imaging techniques, utilising established measurements such as gamma scintigraphy in association with newer techniques including Magnetic Resonance Imaging (MRI).

8.3 Experimental

8.3.1 Materials

Freund's Complete adjuvant was purchased from Bangalore Genei limited, Glutathione was purchased from Sigma chemical co.; St. Louis, MO.

8.3.2 Apparatus

Rat cages, glass syringe (1 ml capacity) with 26 gauge needle, Varnier callipers.

8.3.3 Selection of animals

Male Sprague-Dawley rats weighing 300-350 gms were chosen for scintigraphy studies. No diet restriction was enforced prior to studies. Three rats were chosen for each group.

8.3.4 Adjuvant induced arthritis

Monoarticular arthritis was induced in the left knee joints of the Male Sprague-Dawley rats. Arthritis was induced in left knee joints by injecting 0.1 ml of the Complete Freund's adjuvant through the supra-patella ligament using 27 gauge needle (Vogel, 1997).

8.3.5 Intra-articular injection of celecoxib and its formulations

The samples (celecoxib and its formulations) were injected intra-articularly in the left knee joints of the rats in which arthritis was induced. Samples were administered five days after the induction of arthritis. Two hundred µl of celecoxib solution (CS) or celecoxib loaded chitosan microspheres (CMS) or Albumin microspheres (AMS) or gelatin microspheres (GMS) or Solid lipid nanoparticles (SLN) were injected intra-articularly in the left knee joint to each rat. One group which served as control did not receive any treatment. Three rats were used in each group.

8.3.6 Evaluation of arthritis by measurement of knee diameter

The development of arthritis was monitored regularly by measuring changes in the knee joint diameter using varnier calipers, the mean of three readings being taken with the joint flexed at 90°C. Four days after the induction of arthritis, the diameter of the arthritic joint was 19.4±0.2 mm while that of the control joint was 12.2±0.3mm. The knee joint diameter of each rat was measured at the 3^{rd}, 10^{th} and 18^{th} day after administration of the samples.

8.3.7 Evaluation of arthritis by γ-scintigraphy

Technetium labelled glutathione (Ercan et al, 1994) was used as a radiopharmaceutical to demonstrate arthritic lesions by gamma scintigraphy before therapy. Four days after the induction of arthritis, each rat was administered with 0.2 ml (200 μci) of ^{99m}Tc-glutathione intra-venously through the tail vein. Four hours after the administration of the radiopharmaceutical, static images were obtained from the posterior position with a gamma camera using a LEAP collimator. 100-150 kilo-counts were collected to obtain an image. Regions of interest were drawn over both the arthritic (target) and contralateral (nontarget) knee joints. The right joint which was not made arthritic served as a control. The radioactive count ratios obtained from the equal regions of interest of Arthritic (A) and Control (C) were used to determine the A: C ratios. Evaluation of arthritis was performed by the intravenous injection of ^{99m}Tc-labelled glutathione, as explained before, on 3rd , 10th and 18th day post intra-articular injection of the samples. Four hours after the administration of the radiopharmaceutical, scintigraphic images of rats were obtained as described previously. Regions of interest were created in target and non-target areas, and the radioactivity count ratios (A: C) in each group were determined.

8.3.8 Data analysis

The results are expressed as mean ± S.D. and were analyzed using a Kruskal–Wallis multiple comparison test followed by post-hoc Dunn's test at the significance level of $P<0.05$ and $p<0.1$.

Table 8.1: Knee joint diameters of the different groups before and after treatment

Code	n	Before induction of arthritis	Pre-treated (mm)	Post-treated (3^{rd} day)	Post-treated (10th day) (mm)	Post-treated (18^{th} day) (mm)
CS	3	12.4±0.30	19.7±0.35	16.4±0.56	17.2±1.05	17.8±1.51
CMS	3	14.0±0.75	18.4±0.26	15.6±0.85	15.3±0.52	14.8±1.25
AMS	3	13.4±0.70	17.9±0.44	17.6±0.70	17.1±1.10	17.4±0.92
GMS	3	13.2±0.56	18.1±0.50	17.5±0.72	16.8±0.46	14.7±0.46
SLN	3	12.5±0.40	17.8±0.95	17.7±0.44	17.3±0.66	17.5±1.23
Control	3	14.3±0.46	19.6±1.23	19.7±0.26	19.5±0.60	19.8±0.62

All results are expressed as mean ± S.D.
CS= Celecoxib solution
CMS= Celecoxib loaded chitosan Microspheres
AMS= Celecoxib loaded albumin microspheres
GMS= Celecoxib loaded gelatin microspheres
SLN=Celecoxib loaded solid lipid nanoparticles

Figure 8.1: Knee joint diameters of different groups before and after treatment

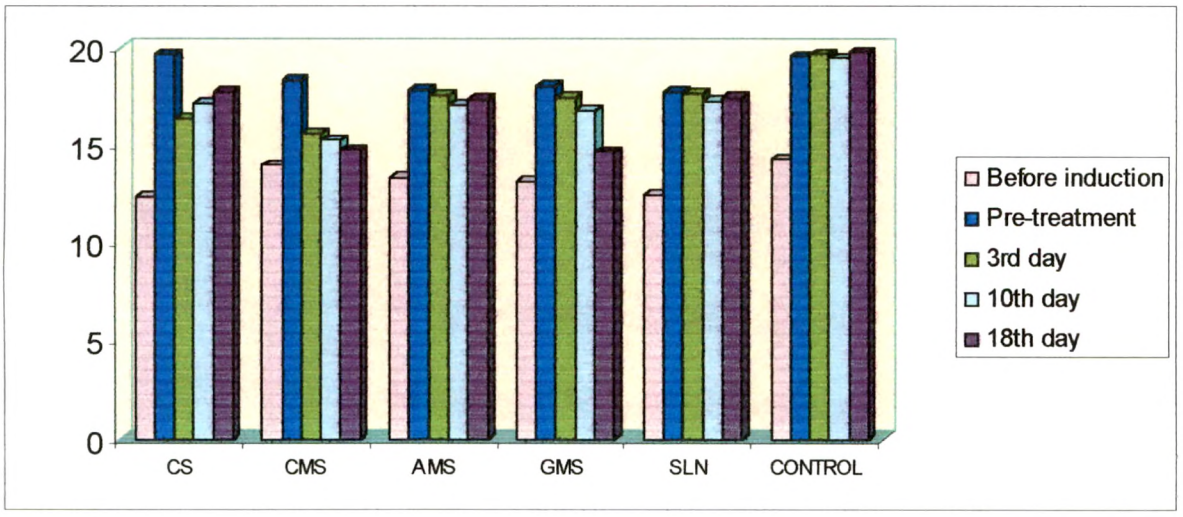

Table 8.2: The radioactivity count ratios (A: C) in target: non-target areas in the different groups before and after treatment

Code	n	Pre-treated	Post-treated (3^{rd} day)	Post-treated (10th day)	Post-treated (18^{th} day)
CS	3	2.54±0.43	1.86±0.45	2.11±0.40	2.28±0.56
CMS	3	3.05±0.30	2.42±0.40	1.91±0.73	1.50±0.31
AMS	3	3.57±0.49	3.16±0.22	3.26±0.16	3.05±0.49
SLN	3	3.62±0.26	3.26±0.21	3.01±0.27	3.16±0.42
GMS	3	3.36±0.36	2.85±0.11	2.37±0.30	1.90±0.31
Control	3	2.27±0.77	3.15±0.56	2.86±0.68	2.78±0.64

All results are expressed as mean ± S.D.
CS= Celecoxib solution
CMS= Celecoxib loaded chitosan Microspheres
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GMS= Celecoxib loaded solid lipid nanoparticles
SLN= Celecoxib loaded gelatin microspheres

Figure 8.2: The radioactivity count ratios (A: C) in different groups before and after treatment

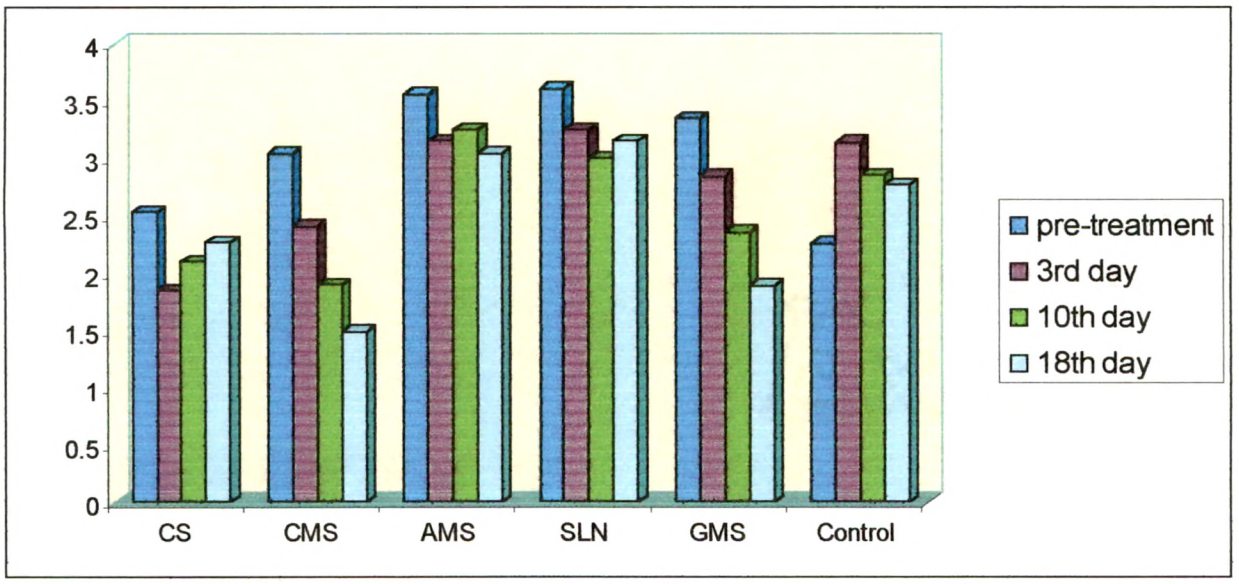
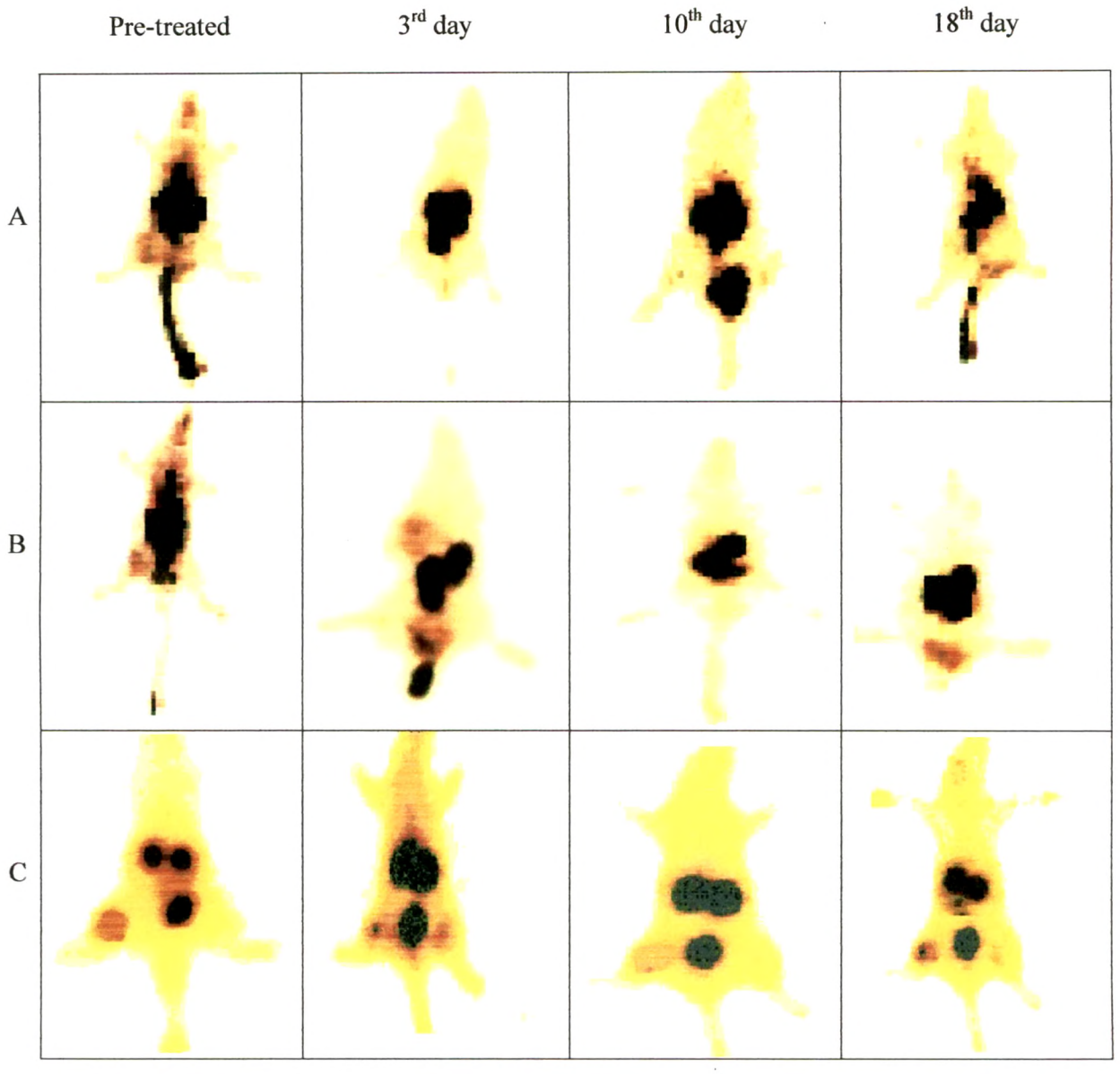


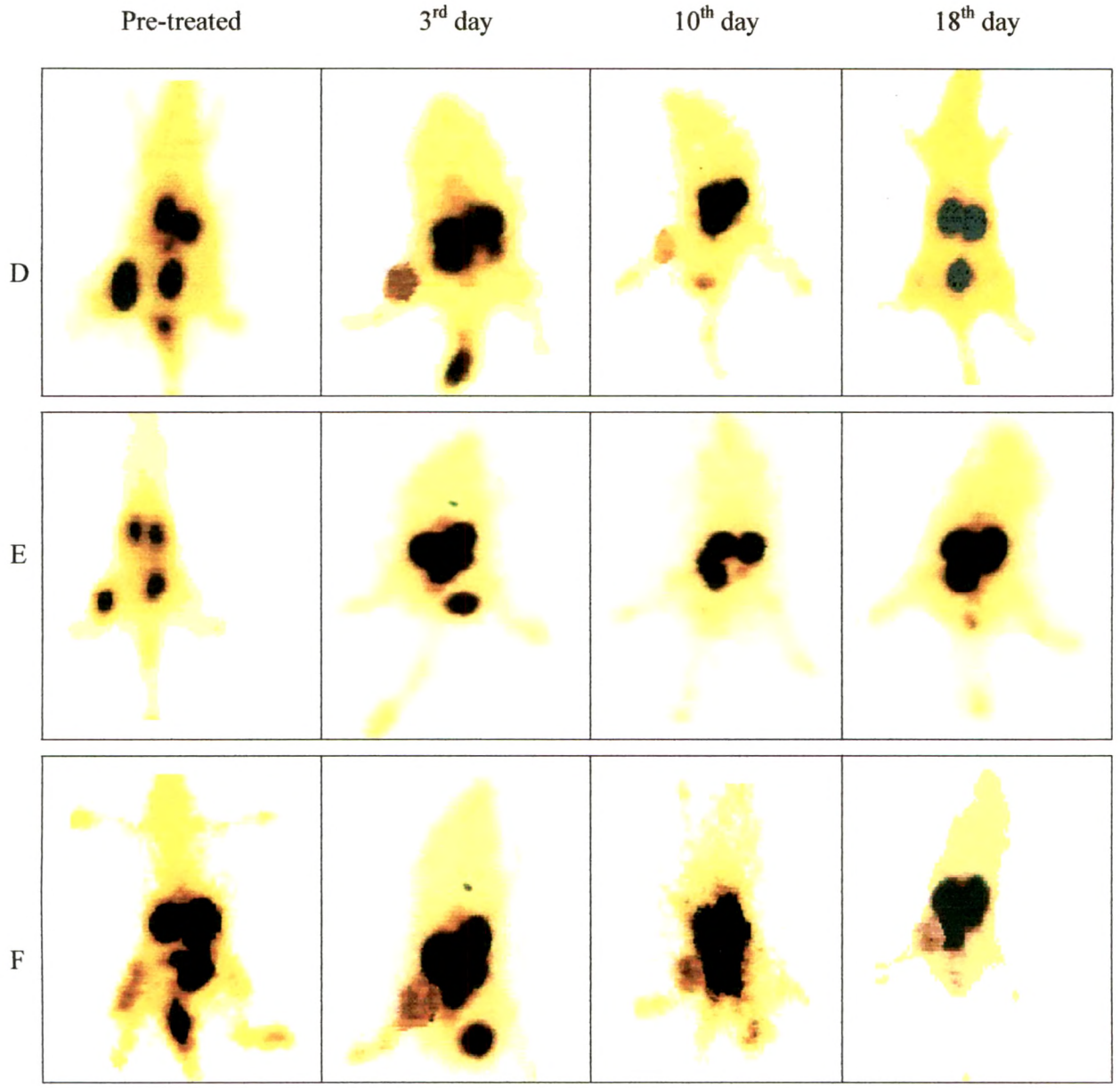
Figure 8.3: γ-Scintigrams of arthritic rats taken 4 hours post administration of ^{99m}Tc-glutathione

A) Celecoxib solution B) Celecoxib loaded chitosan microspheres

C) Celecoxib loaded albumin microspheres D) Celecoxib loaded solid lipid nanoparticles

E) Celecoxib loaded gelatin microspheres F) Control




Pre-treated
3rd day
10th day
18th day
D
E
F

8.4 Results and discussion

Freund's adjuvant was used to induce arthritis in rats as it is reported to produce arthritis closely resembling human rheumatoid arthritis (Vogel, 1997). There is a significant increase in the knee joint diameter of the rats after induction of arthritis as shown in table 8.1 After intra-articular injection of celecoxib, there is a decrease in the knee joint diameter from 19.7mm to 16.4mm at 3rd day after the injection. This indicates a decrease in the inflammation. But this decrease is of a short duration and thus 10th and 18th day post intra-articular injection, there is an increase in the knee joint diameter. There is no significant difference ($p>0.1$) in the knee joint diameters pre-treatment and 18th day post treatment in case of celecoxib solution. In case of albumin microspheres and solid lipid nanoparticles also a similar effect is observed. There is no significant decrease in the knee joint diameters after intra-articular injection of albumin microspheres and solid lipid nanoparticles, indicating that there is no decrease in the inflammation. While in case of chitosan microspheres and gelatin microspheres loaded with celecoxib, there is a gradual decrease in the knee joint diameters at each time interval. This indicates a longer duration of anti-inflammatory effect observed with these formulations.

The use of γ-scintigraphy for evaluation of arthritis was also done as it is a more sensitive method for imaging inflammatory lesions. As shown in figure 8.3, arthritis could be well demonstrated by intra-venous injection of ^{99m}Tc-glutathione. In earlier studies (Bozdag et al, 2001, Tuncay et al, 2000a, Tuncay et al, 2000b), ^{99m}Tc-HIG has been used for the assessment of arthritis in rabbits. In the present study, ^{99m}Tc-glutathione was used as a radiopharmaceutical to demonstrate arthritic lesions in rats. Various radiopharmaceuticals like ^{67}Ga-citrate and proteins such as human immunoglobulin labelled with various radio-isotopes are used for imaging the inflammatory lesions. The basic mechanism behind the accumulation of these agents in the inflammatory lesions is the increased capillary

permeability. These agents are large molecular weight compounds having prolonged blood clearance, which leads to delayed studies for inflammatory lesions. ^{99m}Tc-glutathione, because of its smaller size, diffuses more rapidly into the inflamed tissues achieving higher concentrations earlier. Thus, it proved to be better than ^{99m}Tc-HIG for the assessment of arthritis (Ercan et al., 1994). The labelling efficiency of the ^{99m}Tc-glutathione was determined to be 99% and it was stable for 24 hours. As it can be seen in the figure 8.3, there is no accumulation of the radioactivity in the non-inflamed right joint. This clearly demonstrates that there is a preferential localization of ^{99m}Tc-glutathione in the inflamed region. For quantitative determination of inflammation, regions of interest (ROI) were drawn over both arthritic and normal joints and radioactivity count ratios of target to non-target areas were calculated with standard deviations and mean values. As shown in Table 8.2, the higher values of A: C ratios in all the groups pre-treatment indicate the induction of arthritis and well demonstration of arthritic lesions (Figure 8.3). After intra-articular injection of the CS, CMS and GMS, there is a significant reduction in the A: C ratios indicating a healing effect. But in case of the drug solution (CS), this effect is short lived compared to the microspheres. There is a reduction in the A: C ratios 3 days post treatment in case of CS, CMS as well as GMS. But in case of the drug solution, there is an increase in the A: C ratio at 10th and 18th day post treatment indicating a short duration of effect. On the other hand, in the case of CMS and GMS, the A: C ratio is gradually decreasing from 3rd day to the 18th day indicating a slow release of the drug from the microspheres and a longer duration of anti-inflammatory effect.

In case of SLN and AMS, there is no significant reduction in the A: C ratios 3rd, 10th or 18th post intra-articular injection, indicating that there is no anti-inflammatory effect when celecoxib entrapped in these formulations in injected intra-articularly. The reason behind

this finding may be that the microspheres itself may have caused inflammation when injected intra-articularly. This was confirmed by the histopathology studies which indicated that albumin microspheres did produce some inflammatory changes in the synovium of the rat after intra-articular injection. In the control group which did not receive any drug there is no significant reduction in the A: C ratio which clearly demonstrates that there is no healing effect as the time progresses in absence of the drug. The difference in the A: C ratio at pre-treatment and 18th day post-treatment in case of drug solution is not significant. However, in case of the CS and GMS, this difference is significant indicating an enhanced duration of anti-inflammatory effect of the drug loaded microspheres.

In the present study the effect of the celecoxib solution and celecoxib loaded microspheres and solid lipid nanoparticles after intra-articular administration was studied in arthritic rats. The optimal characteristics of any formulation are the high drug loading and high entrapment efficiency which will lead to less wastage of the drug. The high drug loading of the microspheres would allow the use of lesser quantity of microspheres for injection. Chitosan microspheres and albumin had an entrapment efficiency of around 90%, gelatin microspheres had an entrapment efficiency of around 86% and the solid lipid nanoparticles had an entrapment efficiency of around 99%. Thus all the formulations fulfilled the desired criteria of high entrapment efficiency (>80%).

For intra-articular injection, the particle size of the microsphere is also very important. The particle size should be small enough so that upon exposure to the inflamed synovium, the particles are phagocytosed by the macrophages. According to the work done by previous workers, particles with a size range of 0.0025 to 10μm are readily phagocytosed (Benacereff et al, 1975). However, for maximal phagocytic uptake, the particle size should be less than 5μm (Ratcliffe et al., 1984). The size-dependency of the particulate

carrier for intra-articular delivery system on phagocytosis in rat synovium has been investigated by the earlier workers (Horisawa et al., 2002). They have concluded that the PLGA nanospheres having a size of 265 nm were phagocytosed in the synovium by the macrophages while the microspheres having a size of 26.5μm were not phagocytosed in the macrophages. Thus, nanospheres should be more suitable for the delivery to the inflamed synovial tissue than the microspheres due to their ability to penetrate the synovium. The geometric mean diameter of the chitosan microspheres was around 8μm, that of albumin microspheres was around 5μm, gelatin microspheres around 20μm and solid lipid nanoparticles around 250 nm. Thus out of all the formulations, chitosan microspheres, albumin microspheres and solid lipid nanoparticles had particle size susceptible to phagocytosis by the macrophages of the inflamed synovium. Thus, they are expected to be retained in the inflamed joint for prolonged periods of time, which was confirmed by the biodistribution studies. Gelatin microspheres had a higher particle size, which is not prone to be phagocytosed by the macrophages of the inflamed synovium. But the higher particle size does not allow the clearance of the particles into the circulation and hence the microspheres are expected to remain at the inflamed site for prolonged periods of time. This was confirmed by the biodistribution studies, which indicated that out of all the formulations, gelatin microspheres exhibited highest retention in the inflamed joint after intra-articular injection.

The drug release from the microspheres is also an important parameter in the intra-articular drug delivery system. The drug should be released slowly from the microspheres so that the cartilage is not exposed to a significant level of the drug. Thus it is expected from the microspheres that after intra-articular injection, the drug should be released slowly from the microsphere as long as they remain in the joint. The in-vitro studies indicated that from all the formulations celecoxib is released within 7 days. However, the

in-vivo studies indicate that the drug loaded in microspheres is having an anti-inflammatory effect for a period of over 18 days. The in-vitro results of the release rates cannot be extrapolated to predict the kinetics of release in-vivo. The rate of the drug release may be dampened because of the high viscosity of the synovial fluid and adherence of microspheres to synovium and cartilage. Thus, the release rates in-vivo is expected to be slower than that obtained in-vitro.

Various reports on the use of particulate carrier for intra-articular injection are available in the literature (Bozdag et al, 2001, Brown et al, 1998, Ratcliffe et al, 1984). A comparison between a natural polymer albumin with a synthetic polymer PLGA (50:50) has been made by the earlier workers (Tuncay et al, 2000b) for use as an intra-articular drug delivery system. The workers concluded that the natural polymer BSA is more promising than the synthetic type of polymer PLGA for intra-articular administration of Diclofenac sodium. Hence, an attempt was made to compare the other natural polymers such as chitosan and gelatin and also solid lipid nanoparticles with the natural polymer Bovine serum albumin. Our studies indicated that, compared to bovine serum albumin and solid lipid nanoparticles, gelatin and chitosan microspheres exhibited prolonged anti-inflammatory effect on the inflamed synovium of the rats.

8.5 References

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