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Efficacy of chitosan microspheres for controlled intra-articular delivery of celecoxib in inflamed joints

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Abstract

The use of polymeric carriers in formulations of therapeutic drug delivery systems has gained widespread application, due to their advantage of being biodegradable and biocompatible. In this study, we aimed to prepare celecoxib-loaded chitosan microspheres for intra-articular administration and to compare the retention of the celecoxib solution and chitosan microspheres in the joint cavity. The microspheres were characterized for entrapment efficiency, particle size and surface morphology by scanning electron microscopy. In-vitro drug release studies of microspheres revealed that the microspheres are able to control the release of celecoxib over a period of 96 h. Biodistribution studies of celecoxib and chitosan microspheres were performed by radiolabelling with ^{99m}Tc and injecting intra-articularly in rats. The study indicated that following intra-articular administration the distribution of the drug to the organs, like liver and spleen, is very rapid compared with that of the microspheres. Compared with the drug solution, a 10-fold increase in the concentration of the drug in the joint was observed 24 h post intra-articular injection ($P < 0.005$) when drug was encapsulated in microspheres.

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Introduction

Celecoxib has been recently introduced as a non-steroidal anti-inflammatory drug (NSAID) that exhibits anti-inflammatory, analgesic and antipyretic activity, especially in arthritis. Its mechanism of action is believed to be due to the inhibition of prostaglandin synthesis, primarily via inhibition of cyclo-oxygenase-2 (COX-II). It has been shown that at therapeutic concentration in man, celecoxib does not inhibit the COX-1 isoenzyme (Woolf et al 2000), which is responsible for gastrointestinal bleeding and ulcers.

Celecoxib is thus associated with a lower incidence of gastroduodenal ulcers than other NSAIDs (Ashcroft et al 2001) that are non-specific inhibitors of cyclo-oxygenase. But since COX-II is constitutively present in some organs, like the kidney and brain, and can be induced in other tissues, COX-II-specific inhibitors are not devoid of side effects. Thus, COX-II inhibitors can cause thrombotic cardiovascular disease as well as renal disease (Bing 2003). Achieving a higher concentration of the drug at the arthritic joint and minimizing its distribution to the other tissues would minimize the side effects of the drug. One way of achieving this is to inject the drug intra-articularly, although this is complicated by the fact that the very efficient lymphatic system in the joint clears intra-articular drugs and debris from the synovial cavity (Noble et al 1983; Wallis et al 1987). For example, following intra-articular administration of corticosteroids, suppression of plasma cortisol (Bird et al 1979) and a decrease in number of circulating eosinophils have been observed. Therefore, to achieve a higher concentration in the joint cavity and minimize distribution to other organs, the administration of drug encapsulated in liposomes (Dingle et al 1978; Foong & Green 1988, 1993; Camilleri et al 1993; Monkkonen et al 1995) or microspheres prepared with albumin (Ratcliffe et al 1984, 1987; Tuncay et al 2000a), gelatin/chondroitin sulfate (Brown et al 1998) or polylactic acid/polyglycolic acid copolymer (Tuncay et al 2000b; Bozdag et al 2001; Horisawa et al 2002), has been attempted. The principal requirement of an ideal intra-articular drug delivery system is to retain high concentrations of the drug in the joint cavity for a prolonged period of time and control the release of the drug. Incorporation of drugs within biodegradable polymer

particles has been shown to be effective in improving the retention of drugs within the joint cavity (Ratcliffe et al 1987). It was therefore anticipated that incorporation in biodegradable polymer particles would enhance the efficacy of celecoxib injected into the arthritic joints. Moreover, the increased retention of the drug at the injection site should reduce the potential adverse effects of the drug.

Chitosan, a deacetylated derivative of chitin, a natural polysaccharide found in the shells of crustaceans, is widely used in drug delivery systems (Lim et al 1997; Akbuga & Bergisadi 1999; Shu & Zhu 2001; Banerjee et al 2002). This is because of its excellent biocompatibility, biodegradability and natural abundance of chitin, a natural organic material used to manufacture chitosan (Kumbhar et al 2002). Also it is susceptible to cross-linking by dialdehydes such as glutaraldehyde to various degrees to control the release of the drug (Jameela et al 1998). Thus, the purpose of this study was to prepare celecoxib-loaded chitosan microspheres and to evaluate their in-vitro and in-vivo distribution. The retention of the microspheres in the inflamed joint of rats was evaluated by radiolabelling the drug and the microspheres and determining the radioactivity present in the joints at specific time intervals. The biocompatibility of the prepared microspheres with the synovial tissue was also investigated by histopathological examination.

Materials and Methods

Chemicals

Celecoxib was a gift from Sun Pharmaceutical Advanced Research Centre. Chitosan (MW 6.524×10^5, degree of deacetylation 81.5%) was kindly provided by Central Fisheries Technology Limited (Cochin). The cross-linking agent was glutaral (25%) (E. Merck India Ltd). Tween-80 was purchased from S.D. Fine Chem. Ltd (Mumbai). Diethylene triamine penta-acetic acid (DTPA) and stannous chloride ($SnCl_2.2H_2O$) were purchased from Sigma Chemical Co. (St Louis, MO). Sodium pertechnetate eluted from molybdenum-99 by the solvent extraction method was procured from Regional Center for Radiopharmaceuticals (Northern Region), Board of Radiation and Isotope Technology (Delhi, India). Complete Freund's adjuvant was purchased from Bangalore Genei Ltd. All other chemicals and solvents were of analytical grade and were used without further purification.

Preparation of microspheres

Chitosan microspheres were prepared using an emulsion chemical cross-linking technique (Wang et al 1996). A weighed amount of chitosan (3% w/w) was dissolved in 2% acetic acid. Tween-80 was added at a concentration of 2% w/w. Celecoxib was finely triturated in a mortar. Finely powdered celecoxib (10 mg) was added to the above solution and sonicated to obtain a uniform dispersion. One millilitre of this dispersion was injected into a mixture of 20 mL heavy liquid paraffin and 0.5 mL Span-85 while stirring at 2500 rev min^{-1}. Stirring was continued for 10 min to obtain a water-in-oil (w/o) emulsion. One millilitre of glutaral (25%) was added into the emulsion to cross-link the chitosan present in the internal phase of the emulsion. Stirring was continued for 3 h. Microspheres were then separated by centrifugation at 5000 rev min^{-1} and then washed with 30 mL petroleum ether to remove the liquid paraffin. The microspheres were then suspended in 10 mL of 5% (w/v) sodium bisulfite solution and stirred on a magnetic stirrer for 10 min to remove the residual glutaral. Finally, the microspheres were washed with 100 mL water and dried at room temperature for 24 h. The prepared microspheres were tested for the residual glutaral by the hydroxylamine hydrochloride method (USP 1990). The microspheres were then stored in a desiccator till further use.

Characterisation of microspheres

Entrapment efficiency

A weighed amount of the chitosan-loaded microspheres was suspended in 0.1 M hydrochloric acid and allowed to stand for 24 h. The dispersion was then shaken with methylene chloride to extract celecoxib. The organic extract was evaporated to dryness and the residue dissolved in methanol. The absorbance of the resulting solution was measured at 250 nm on a Shimadzu 1601 UV-VIS spectrophotometer to determine the amount of celecoxib present in the microspheres.

Particle size

The particle size distribution of the microspheres was determined by laser light scattering on a Malvern Particle Size Analyzer (Malvern Master Sizer 2000; SM, UK). The microspheres were added to the sample dispersion unit containing the stirrer and stirred to reduce the aggregation between the microspheres, and laser obscuration range was maintained at 15–20%. The mean particle size was measured after performing the experiment in triplicate.

In-vitro drug release

Drug release from the microspheres was determined using phosphate buffer, pH 7.4, containing 2% w/w Tween-80 as the release medium. Microspheres were suspended in 50 mL of the dissolution medium in a 100-mL glass vial and stirred on a magnetic stirrer at 50 rev min^{-1} in a thermostated bath at 37°C. Samples (2 mL) were withdrawn at appropriate time intervals and centrifuged at 5000 rev min^{-1}. Supernatants were diluted suitably and absorbance of the resulting solution was measured at 250 nm using the dissolution medium as blank. The residue was re-dispersed in 2 mL of the fresh dissolution medium and replaced back into the vial.

Surface morphology of microspheres

Scanning electron microscopy of the celecoxib-loaded microspheres was carried out to examine the surface morphology. The microsphere powder was coated with gold and then kept in the sampling unit as a thin film. Photographs were taken at 1000 magnification using a

Jeol Scanning Electron Microscope (JSM-5610LV SEM; Jeol, Japan).

Radiolabelling of celecoxib and its microspheres
Celecoxib and the celecoxib-loaded microspheres were labelled with ^{99m}Tc by direct labelling method as described earlier (Richardson et al 1977). Briefly, 1 mL of ^{99m}Tc (2 mCi mL^{-1}) was mixed with 60 μL of stannous chloride solution in 10% acetic acid (1 mg mL^{-1}) for the drug solution and with 100 μL for the microspheres and the pH was adjusted to 6.5 using 0.5 M sodium bicarbonate solution. To this mixture, 1 mL of the celecoxib solution (2 mg mL^{-1}) or the microsphere suspension (30–40 mg mL^{-1}) was added and incubated for 10 min at room temperature. The quality control was performed as per the method described earlier (Theobald 1990). The effect of pH on the labelling efficiency of ^{99m}Tc–celecoxib/microspheres was studied by determining the labelling efficiency, keeping the other variables constant and changing the pH.

The effect of stannous chloride concentration on the labelling efficiency was also studied by using different concentrations of stannous chloride. To study the effect of incubation time on the labelling efficiency, the radiolabelled complexes were incubated for various time periods keeping the other variables constant.

Labelling efficiency
The radiochemical purity of ^{99m}Tc with celecoxib or the microspheres was estimated by ascending instant thin-layer chromatography (ITLC) using silica-gel-coated fibre sheets (Gelman Sciences Inc., Ann Arbor, MI). The ITLC was performed using 100% acetone or 0.9% saline as the mobile phase. Around 2–3 μL of the radiolabelled 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 ($R_f = 0.9$), was determined. The reduced/hydrolysed (R/H) technetium, along with the labelled complex, remained at the point of application. The amount of R/H ^{99m}Tc (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 labelled 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 radiolabelled celecoxib, as well as microspheres, was calculated.

Stability study of ^{99m}Tc–celecoxib/microsphere complex
The stability of the radiolabelled complex was determined in-vitro using 0.9% sodium chloride and serum by ascending thin-layer chromatography. The radiolabelled complex (0.1 mL) was mixed with 1.9 mL 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.

Transchelation with DTPA
To check the stability and strength of the binding of ^{99m}Tc with celecoxib or celecoxib-loaded microspheres, 0.5 mL of 1.0 mM solutions of DTPA in saline were taken in separate 5-mL vials. To these vials, 0.1 mL of the radiolabelled celecoxib/microspheres was added. After brief mixing, each vial was incubated for 1 h at 37°C. As a control, 0.5 mL of saline was mixed with 0.1 mL 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 ($R_f = 0.8$–1) from the ^{99m}Tc–celecoxib/microsphere complex, which remained at the point of application ($R_f = 0$).

In vivo studies

Adjuvant induced arthritis
Male Sprague-Dawley rats, 300–350 g, were used for in-vivo studies. Mono-articular arthritis was induced in left knee joints of the rats by injecting 0.1 mL of Complete Freund's adjuvant through the supra-patella ligament using a 27-gauge needle. 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°. Four days after the induction of the arthritis, the diameter of the arthritic joint was 19.4 ± 0.2 mm while that of the control joint was 12.2 ± 0.3 mm.

Extra-articular distribution
All animal experiments were approved by the Animal Ethics Committee of the institution. Biodistribution of the ^{99m}Tc-labelled celecoxib or microspheres was studied in arthritic Sprague-Dawley rats, 300–350 g. Four days after the induction of the arthritis, 200 μL (200 μCi) of the radiolabelled preparation was injected intra-articularly into the left knee joint of each rat. Groups of three rats per time point were used in the study. The rats were sacrificed at different time intervals and blood was obtained by cardiac puncture. Subsequently tissues (heart, lung, liver, kidney, spleen, intestine, stomach) were removed. The knee joints, both the left and the right were exposed and cut into fragments. All the tissues were washed with normal saline, blotted dry, weighed and their radioactivity was measured in a well-type gamma scintillation counter. To correct for physical decay and to calculate radiopharmaceutical uptake in each organ as a fraction of the injected dose, samples of the injectate, containing 1% of the injected dose, were counted simultaneously at each time point.

Blood kinetics
The clearance of the ^{99m}Tc–celecoxib/microspheres into the systemic circulation after intra-articular administration was studied in arthritic rabbits, 3–3.5 kg. Arthritis was induced

in the left knee joints of the rabbits by injecting 0.5 mL of Complete Freund's adjuvant. Assessment of arthritis was done by measuring the knee joint diameter by means of Varnier Calipers. The diameter of the arthritic joint after 4 days of induction was 33.5 ± 0.4 mm while that of the control joint was 25.4 ± 0.3 mm. The labelled preparation (500 μL, 500 μCi) was injected intra-articularly into the left knee joints of each rabbit of known weight. Blood was withdrawn at different time intervals from the marginal ear vein. The radioactivity was measured in a well-type gamma ray counter (Gamma ray spectrometer, Type GRS23C; Electronics Corporation of India Ltd). The blood was weighed and the radioactivity present in the whole blood was calculated by keeping 7.3% of the body weight as the total weight of the blood (Wu et al 1981).

Evaluation of biocompatibility

The potential toxicity of the microspheres to the synovium was evaluated by histopathological studies. Plain chitosan microspheres (8–10 mg) were suspended in 0.2 mL saline and injected into the left knee joint, 0.2 mL saline being injected into the right knee joint as control. Three days after the injection, the rats were sacrificed and the joints were isolated. They were fixed in buffered formalin and embedded in paraffin wax. Sections (5 μm) were cut, stained with eosin and haemotoxylin and evaluated for the inflammatory changes.

Statistical analysis

The results are expressed as mean $\pm$ s.d. and were analysed using a Kruskal–Wallis multiple comparison test followed by post-hoc Dunn's test at the significance levels of $P < 0.05$ and 0.005. The effect of DTPA on the % radiolabelled celecoxib or celecoxib-loaded microspheres and the blood kinetics data were anlaysed using a Mann–Whitney *U*-test.

Results and Discussion

Microsphere characteristics

Chitosan is chosen as a matrix material because of its biocompatibility, low cost and bioadhesiveness. Also it can be suitably cross-linked to control the release of the drug. The method used produced the microspheres with high entrapment efficiency.

The entrapment efficiency, with respect to celecoxib, of the chitosan microspheres was found to be around 95% and was mainly dependent on the concentration of chitosan used, the emulsifier concentration and the stirring rate. Increasing the concentration of chitosan from 2 to 3% w/w increased the entrapment efficiency from 75 to 95%. As the emulsifier concentration increased from 2 to 5% the entrapment efficiency decreased from 95 to 80%. This is because celecoxib, being a lipophilic drug, is soluble in the external phase of the w/o emulsion at a high concentration of Span-85.

The stirring speed also had an influence on the entrapment efficiency. Increasing the stirring speed from 2500 to 4000 rev $\min^{-1}$ decreased the entrapment efficiency from 95 to 87%.

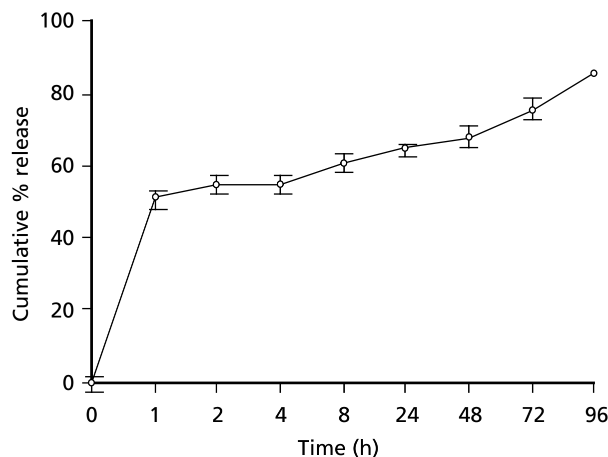

Figure 1 In-vitro release profile of celecoxib-loaded chitosan microspheres. Each value is a mean of triplicate results and error bars represent s.d.

The particle size of the optimum formulation was found to be around 8 μm and was found to be dependent mainly on the concentration of the emulsifier and the stirring speed. Increasing the concentration of the emulsifier from 1 to 2% decreased the particle size from 15 to 8 μm. Increasing the stirring speed from 1000 to 2500 rev $\min^{-1}$ decreased the particle size from 14 to 8 μm.

The drug release from the microspheres was found to be dependent on various factors, such as the concentration of the chitosan, concentration of the cross-linking agent and duration of cross-linking. Increasing the concentration of cross-linking agent and duration of cross-linking leads to a decrease in the rate of drug release. The release profile of the optimized formulation is shown in Figure 1. Chitosan microspheres were able to control the release of drug over a period of 96 h. However, a burst effect was observed in all the formulations. In general, around 40% of the drug was released in the first hour, followed by slower release over a period of 96 h. This is in concurrence with previous reports (Denkbas et al 1999).

The glutaral employed for cross-linking chitosan was removed by washing with water, until the residual content was insignificant. However, the residual glutaral was removed by reacting with sodium bisulfite (Thanoo et al 1993). The nucleophilic addition of bisulfite across the pi-bond of the carbonyl group produces a water-soluble sodium salt of an organic sulfite, which can be easily removed by water washing. The residual glutaral, determined by reaction with hydroxylamine hydrochloride (USP 1990), was found to be less than 5 ppm.

The scanning electron microscopy studies revealed that the microspheres were spherical in shape, having a somewhat rough surface (Figure 2).

Radiolabelling of microspheres

Radiolabelling has proven to be quite useful in following the fate of injected microspheres in-vivo and also as

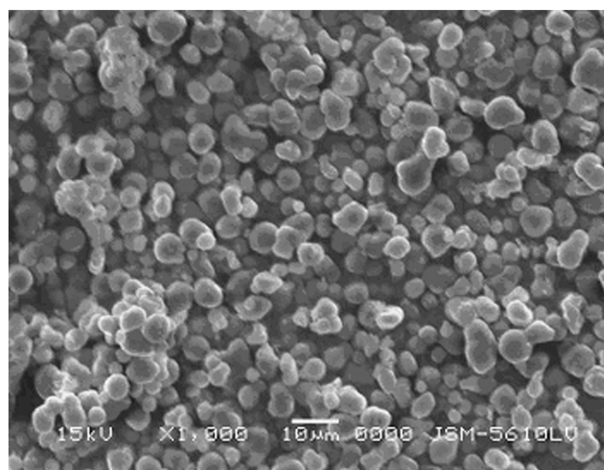

Figure 2 Scanning electron micrograph of celecoxib-loaded chitosan microspheres using 1000× magnification.

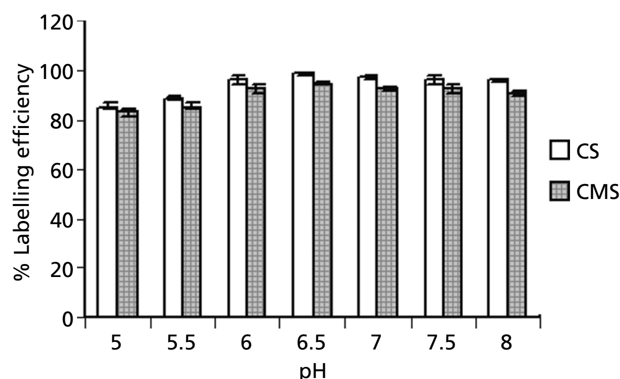

Figure 3 Effect of pH on the labelling efficiency of celecoxib (CS) and its microspheres (CMS). Each value is a mean of triplicate results and error bars represent s.d.

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, 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 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 and chitosan both contain a free NH_2 group, labelling with ^{99m}Tc was possible. Celecoxib and its microspheres were labelled with high efficiency by the direct labelling 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 TcO_4^- 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 optimised by taking three factors into consideration (pH of the complex, stannous chloride dihydrate concentration and incubation time). Figure 3 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 the microspheres. With further increase in the pH, there was a decrease in the labelling efficiency. This indicates that there was a significant effect ($P < 0.05$) of pH on the labelling efficiency of celecoxib and celecoxib-loaded microspheres. Dunn's post-hoc test showed that this effect was highly significant between pH 5.5 and 6.5.

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 Table 1. The labelling efficiency 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 the microspheres, an increase in the concentration of stannous chloride from 30 to 100 μg led to a significant increase ($P < 0.05$) in the labelling efficiency

Table 1 Effect of stannous chloride concentration on the radiolabelling (with ^{99m}Tc) efficiency of celecoxib and its microspheres

$SnCl_2.2H_2O$ (μg)	Celecoxib solution			Celecoxib microspheres		
	% 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
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
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
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

All the values are expressed as mean ± s.d., n = 3.

Table 2 Effect of incubation time on the labelling efficiency of celecoxib and its microspheres

Time of incubation (min)	% Radiolabelled	
	Celecoxib	Celecoxib microspheres
0	92.45 ± 1.22	92.73 ± 1.20
5	95.63 ± 1.04	93.45 ± 1.24
10	98.65 ± 1.01	94.60 ± 0.74
20	98.26 ± 1.72	94.12 ± 1.55
30	97.84 ± 0.41	93.81 ± 1.12

All the values are expressed as mean ± s.d., n = 3.

from 70.82 to 94.60%. Further increase in the stannous chloride concentration led to a decrease in the labelling efficiency. Also, in both the drug and the microspheres, when a lower concentration of stannous chloride was used, the remaining activity was as free TcO_4^- while at higher concentrations of stannous chloride, amount of radiocolloids increased.

The incubation time required for high labelling efficiency was found to be 10 min for celecoxib and its microspheres (Table 2). Further increase in the incubation time did not increase the labelling efficiency considerably. There was a significant difference ($P < 0.05$) in the labelling efficiency of celecoxib and chitosan microspheres when incubated for different time intervals.

In-vitro stability

The stability of the labelled celecoxib and celecoxib-loaded microspheres was studied in saline and serum at 37°C (Table 3). 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 microspheres 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 4, 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 radiolabelled celecoxib or microspheres between the control and DTPA group.

Table 4 Transchelation of the radiolabelled complexes with DTPA

	% Radiolabelled	
	Control	DTPA
Celecoxib–^{99m}Tc complex	97.56 ± 1.21	95.35 ± 1.36
Microspheres–^{99m}Tc complex	94.33 ± 1.25	91.46 ± 0.85

All the results are expressed as mean ± s.d., n = 3.

Table 3 In-vitro stability of ^{99m}Tc-labelled celecoxib and ^{99m}Tc-labelled celecoxib microspheres in physiological saline and in serum at 37°C

Time (h)	% Radiolabelled			
	In saline		In Serum	
	Celecoxib	Microspheres	Celecoxib	Microspheres
0	98.65 ± 1.01	94.60 ± 0.74	98.48 ± 1.43	94.34 ± 0.45
0.25	98.40 ± 0.58	94.57 ± 0.82	98.30 ± 1.17	94.30 ± 1.25
0.5	98.32 ± 1.46	94.41 ± 1.33	97.56 ± 1.18	94.13 ± 1.27
1	97.81 ± 1.57	94.30 ± 1.03	97.14 ± 1.33	93.24 ± 0.91
2	97.46 ± 0.76	93.66 ± 0.69	96.82 ± 0.82	93.75 ± 1.12
4	97.20 ± 1.14	93.14 ± 1.22	96.14 ± 1.42	92.46 ± 1.44
24	96.65 ± 0.93	92.72 ± 1.23	94.50 ± 0.80	92.13 ± 1.00

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 data are expressed as mean ± s.d., n = 3.

Clearance from the joint into the blood

The radioactivity present in the blood at various time intervals, after intra-articular injection of the drug solution, as well as microsphere suspension, is shown in Figure 4. Because of the rapid equilibration between the synovial fluid and plasma, clearance of the drug solution from the joint results in the release of appreciable levels of drug into the systemic circulation. Thus, the clearance of the celecoxib solution from the joint was much faster than the celecoxib-loaded microspheres. Entrapment of the drug in the

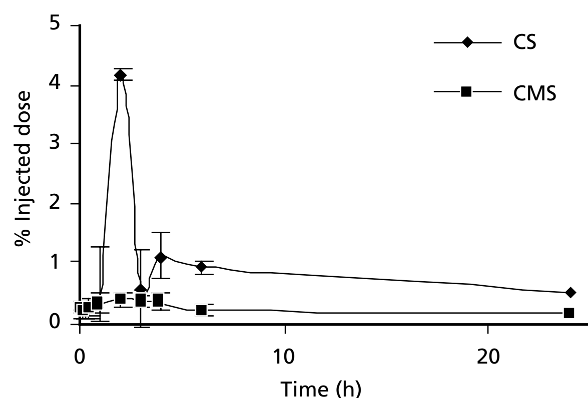

Figure 4 Blood kinetics of ^{99m}Tc–celecoxib (CS) and ^{99m}Tc–celecoxib microspheres in rabbits. The rabbits were administered intra-articularly with 500 μL of ^{99m}Tc-labelled complexes and the radioactivity in the blood was measured at different time intervals. Each value is a mean of triplicate results and the error bars represent s.d.

microspheres led to a delay in this clearance, as well as minimizing the exposure of the cartilage to significantly higher concentrations of the drug. Thus the peak blood concentration of the drug occurred 2 h after intra-articular injection of the drug solution. Similar results were obtained by previous workers (Bird et al 1977; Wigginton et al 1980). The peak plasma concentration of the drug was detected 1–2 h after the intra-articular injection of methotrexate solution (Wigginton et al 1980). Two hours after intra-articular injection, the percent radioactivity present in the blood when microspheres were injected, was almost one-tenth of that of the celecoxib solution. Thus, there is 10-fold increase in the joint concentration of the drug when encapsulated in the microspheres, compared with the drug solution. The Mann–Whitney *U*-test indicated that a significant difference ($P < 0.05$) was observed between the blood concentrations of celecoxib and microspheres, at different time points.

Extra-articular distribution of ^{99m}Tc–celecoxib and ^{99m}Tc–microspheres

The bio-distribution of ^{99m}Tc–celecoxib and ^{99m}Tc–microspheres (loaded with celecoxib) after 4 h and 24 h is shown in Table 5. The percent radioactivity is given per gram of the tissue. Blood was obtained by cardiac puncture, weighed and radioactivity present in the whole blood was calculated by keeping 7.3% of the body weight as total blood weight. Various organs or tissues, like lungs, liver, kidney, spleen, stomach and the inflamed, as well as non-inflamed, joints, were isolated and the radioactivity was determined. In the case of ^{99m}Tc–celecoxib, the radioactivity present in the whole organ/tissue 4 h post injection were found as follows: blood 0.294%, liver 4.4%, spleen 0.185%, lungs 0.192%, kidney and inflamed joint 0.615%. With ^{99m}Tc–microspheres, the major amount of the injected activity remained at the arthritic joint, 9% of the injected dose (the weight of the inflamed joint was around 15 g) being recovered 4 h post intra-articular injection and 5.2% of the injected dose being recovered after 24 h. Thus, a 15-fold increase in percent radioactivity in the inflamed joint was observed 4 h ($P < 0.005$) post intra-articular injection and an almost 10-fold increase 24 h ($P < 0.005$) after the injection. The radioactivity present in the liver was almost 500 times higher for the drug solution, compared with the microspheres ($P < 0.005$), while the radioactivity present in the spleen following drug solution administration was almost 160 times higher than when microspheres were injected ($P < 0.005$). This indicates the rapid clearance of the drug solution from the joint and its extra-articular distribution. Thus, intra-articular injection of the microspheres resulted in greater reduction in distribution of drug to the organs such as liver and spleen. Hence the potential side effects of the drug are expected to be reduced.

The biocompatibility of the microspheres was studied by injecting the plain microspheres in the joint cavity and examining the joints by histopathology. As shown in Figure 5, no inflammatory infiltrates were observed following the intra-articular administration. The microspheres did not produce gross inflammatory changes in the synovium after intra-articular injection. Thus, chitosan microspheres are biocompatible with the synovial cavity and can be used intra-articularly.

Conclusion

The feasibility of using a biodegradable drug delivery system has been investigated for intra-articular administration. Chitosan microspheres were produced with high entrapment efficiency. The geometric mean diameter of the microspheres was found to be around 8 μm. Being a particulate matter, the clearance of the microspheres from the joint is reduced, resulting in improved retention of the

Table 5 Bio-distribution of ^{99m}Tc-labelled celecoxib and ^{99m}Tc-labelled celecoxib microspheres in Sprague-Dawley rats after intra-articular administration

Organ/tissue	% injected dose/gram of organ or tissue			
	4 h		24 h	
	Celecoxib	Microspheres	Celecoxib	Microspheres
Blood	0.014 ± 0.008	0.008 ± 0.0060	0.052 ± 0.005	0.001 ± 0.0004
Heart	0.005 ± 0.0010	0.001 ± 0.0006	0.002 ± 0.001	0.007 ± 0.0042
Liver	0.440 ± 0.0075	0.008 ± 0.0030	0.418 ± 0.066	0.0003 ± 0.0001
Spleen	0.324 ± 0.0500	0.002 ± 0.0050	0.255 ± 0.050	0.016 ± 0.0073
Kidney	0.048 ± 0.0140	0.041 ± 0.0130	0.030 ± 0.010	0.013 ± 0.0053
Lung	0.130 ± 0.0100	0.101 ± 0.0400	0.101 ± 0.027	0.158 ± 0.0326
Intestine	0.002 ± 0.0010	0.001 ± 0.0008	0.002 ± 0.001	0.003 ± 0.0011
Stomach	0.026 ± 0.0060	0.022 ± 0.0070	0.013 ± 0.006	0.015 ± 0.0050
Non-inflamed joint	0.007 ± 0.0020	0.006 ± 0.0020	0.002 ± 0.001	0.002 ± 0.0010
Inflamed joint	0.041 ± 0.0095	0.601 ± 0.0743	0.035 ± 0.004	0.327 ± 0.0450

The rats were administered with 0.2 mL (200 μCi) of the labelled complex intra-articularly and were sacrificed 4 h and 24 h post injection. Each value is mean ± s.e. of 3 rats.

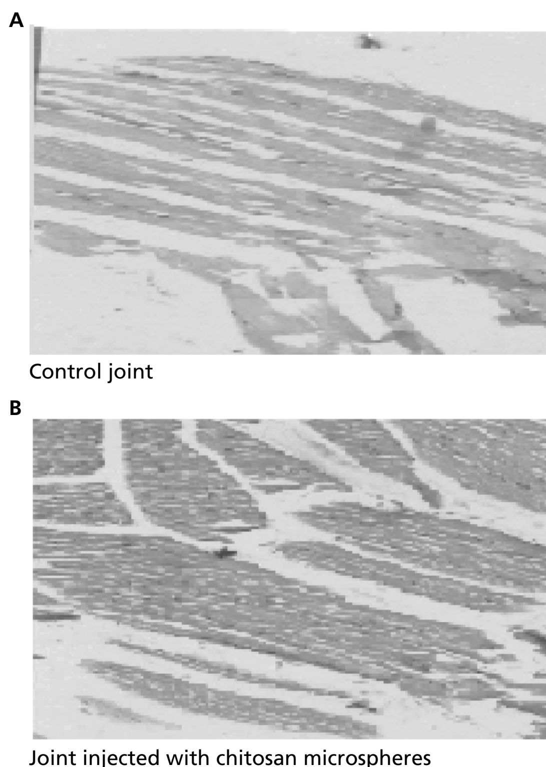

Figure 5 Histopathology of the rat joint. The left joints were injected with a suspension of plain chitosan microspheres and histopathology studies were performed 3 days after the injection to evaluate the biocompatibility of the microspheres (B). The right joint served as a control joint (A).

drug in the joint cavity and minimizing its distribution to the other organs. Biocompatibility study of the prepared microspheres indicated that chitosan is non-inflammatory to the synovium. The improved retention and biocompatible nature implies that the microspheres should be suitable as an intra-articular drug delivery system.

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