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ORIGINAL ARTICLE

Development of gelatin microspheres loaded with diclofenac sodium for intra-articular administration

Muniyandy Saravanan[1], Kesavan Bhaskar[2], Gomathinayagam Maharajan[3], and Kalathil Sadasivan Pillai[4]

[1]*Medicine and Health Sciences, Monash University, Selangor Darul Ehsan, Malaysia,* [2]*Department of Pharmaceutics, Vels University, Chennai, India,* [3]*Department of Physiology, Chettinad University, Chettinad Health City, Chennai, India, and* [4]*Frontier Tissueline Pvt. Ltd, Chennai, India*

Abstract
We have previously reported on the targeting of diclofenac sodium in joint inflammation using gelatin magnetic microspheres. To overcome complications in the administration of magnetic microspheres and achieve higher targeting efficiency, the present work focuses on the formulation of gelatin microspheres for intra-articular administration. Drug-loaded microspheres were prepared by the emulsification/cross-linking method, characterized by drug loading, size distribution, scanning electron microscopy (SEM), Fourier transform infrared (FT-IR) spectroscopy, differential scanning calorimetry (DSC), X-ray diffraction (XRD), gas chromatography, and *in vitro* release studies. The targeting efficiency of microspheres was studied *in vivo* in rabbits. The microspheres showed drug loading of 9.8, 18.3, and 26.7% w/w with an average size range of 37–46 μm, depending upon the drug–polymer ratio. They were spherical in nature and free from surface drug as evidenced by the SEM photographs. FT-IR, DSC, and XRD revealed the absence of drug–polymer interaction and amorphous nature of entrapped drug. Gas chromatography confirms the absences of residual glutaraldehyde. The formulated microspheres could prolong the drug release up to 30 days *in vitro*. About 81.2 and 43.7% of administered drug in the microspheres were recovered from the target joint after 1 and 7 days of postintra-articular injection, respectively, revealing good targeting efficiency.

Keywords: *Targeted delivery; diclofenac sodium; gelatin microspheres; intra-articular administration; depot injection*

Introduction

Diclofenac sodium is one of the few approved nonsteroidal anti-inflammatory drugs available for parenteral administration to treat inflammatory arthritis. Gastric ulcers, gastrointestinal bleeding, blood dyscrasias, and anaphylaxis are potential life-threatening side effects of diclofenac sodium (Carson et al., 1990). We have previously reported (Saravanan et al., 2004; 2008b) about the possibility of targeting diclofenac sodium using gelatin magnetic microspheres. Intra-arterial administration and application of strong magnetic field at the target site are potential practical problems associated with clinical application of magnetic microspheres. To overcome the problems associated with magnetic targeting, in the present study, gelatin microspheres were formulated to localize diclofenac sodium by direct injection into an arthritic joint (compartmental targeting), where the drug will be released slowly to elicit its action.

Intra-articular delivery of drug-loaded microspheres has been developed by many researchers (Ratcliffe et al., 1987; Pavanetto et al., 1994; Tunçay et al., 2000a; 2000b; Bozdag et al., 2001) for targeting drugs into arthritic joint and to achieve a prolonged therapeutic effect. Recently, Larsen et al. (2008) reviewed the potential application of intra-articular depot formulation. The drugs that are administered intra-articularly have a poor biological half-life as they escape from the synovium into the systemic circulation; therefore, it is necessary to administer them in an immobilized form using suitable carriers. Larsen et al. (2008) listed various drugs and carriers used in intra-articular delivery, and from the literature, it is

Address for Correspondence: Muniyandy Saravanan. E-mail: msaravanan72@hotmail.com

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evident that particle size and carrier type are the most important factors in achieving higher level of targeting. In general, particles less than 5 microns had poor retention in the joint and showed less sustained action.

There are few articles (Brown et al., 1998; Larsen et al., 2008) available pertaining to intra-articular delivery of drugs using gelatin microspheres. Microspheres formulated using gelatin and albumin showed greater compatibility than the microspheres of synthetic polymers when injected intra-articularly (Ratcliffe et al., 1984). In our previous work, we have formulated and analyzed albumin and gelatin magnetic microspheres (Saravanan et al., 2008a) for stability and consistency in terms of their release kinetics. Gelatin microspheres were found to be more stable than albumin microspheres.

After analyzing compatibility and stability, in the present work, gelatin was selected as the carrier of choice, and microspheres loaded with diclofenac sodium were prepared by emulsification–glutaraldehyde cross-linking method. The formulated microspheres were systematically characterized by drug content, encapsulation efficiency, size distribution, scanning electron microscopy (SEM) for shape and surface characters, and *in vitro* release studies. The formulated microspheres were analyzed by Fourier transform infrared spectroscopy (FT-IR), differential scanning calorimetry (DSC), and X-ray diffraction (XRD) to find out the physical nature of entrapped drug in the microspheres. The *in vivo* targeting efficiency of microspheres after intra-articular administration was studied in healthy rabbits.

Materials and methods

Materials

Gelatin, type B, 300 Bloom strength was purchased from Sigma Chemicals, St. Louis, MO. Diclofenac sodium was a gift from MARAL, Chennai, India. Anhydrous ether, isopropyl alcohol, toluene, span 80, and glutaraldehyde were purchased from S.D. Fine Chemicals Ltd, Boisar, India, and sesame oil (Idhayam) was purchased from Food World, Chennai, India. All other reagents used were of analytical grade.

Preparation of gelatin microspheres loaded with diclofenac sodium

As shown in Table 1, the required quantity of gelatin was dissolved in 3 ml of phosphate buffer (pH 7.4) preheated to 60°C. The specified quantity of diclofenac sodium was dissolved separately in 3 ml of phosphate buffer (pH 7.4) preheated to 60°C and added to the gelatin solution that was maintained at 60°C. Then the mixture was added dropwise to 100 ml of sesame oil with 1% w/v Span 80 preheated to 60°C and emulsified by stirring with the help of a hand blender (5000 rpm/3 min). Then the stabilized emulsion was allowed to cool and stirred at room temperature using a mechanical stirrer (~1000 rpm; Remi, Mumbai, India). Ten milliliters of glutaraldehyde-saturated toluene solution were added dropwise and the stirring was continued at room temperature for 6 h. The cross-linked microspheres were collected by filtration using Whatman filter paper (no. 41). After filtration, the microspheres were washed with anhydrous ether to remove sesame oil. Then it was washed with 3 × 10 ml of 5% w/v sodium metabisulphite, 2 × 10 ml water, and 2 × 10 ml of isopropyl alcohol. After washing, the microspheres were dried at 45°C, transferred to glass vials and stored in a desiccator.

Determination of drug loading and encapsulation efficiency

Drug-loaded microspheres (100 mg) were digested with 10 ml of 1N sodium hydroxide at room temperature for 12 h. After filtration and suitable dilution, diclofenac sodium present in the solution was determined (Saravanan et al., 2004) at 277 nm using a UV visible spectrophotometer (Shimadzu 1601; Shimadzu, Kyoto, Japan). Drug loading in the microspheres was estimated by using the formula:

$$L = Q_m / W_m \times 100$$

where L is the percentage loading of microspheres, Qm is the quantity of the diclofenac sodium present in Wm g of microspheres. The amount of diclofenac sodium

Table 1. Physical and chemical parameters of gelatin microspheres loaded with diclofenac sodium.

Batch no.	Gelatin (mg)	Diclofenac sodium (mg)	Yield[a] (mg)	Percentage of drug loading: Theoretical	Percentage of drug loading: Actual[a]	Percentage of entrapment[a]	Percentage of encapsulation[a]	Average particle size µm (n = 300) (volume–surface mean)
GM_0	1500	—	1466 ± 82	—	—	—	—	31.01
GM_1	1500	180	1630 ± 43	10.7	9.8 ± 0.9	91.6 ± 8.4	88.7 ± 8.2	37.89
GM_2	1500	380	1770 ± 52	20.2	18.3 ± 1.3	90.6 ± 6.4	85.2 ± 6.1	40.62
GM_3	1500	650	1980 ± 65	30.2	26.7 ± 1.8	88.4 ± 5.9	81.3 ± 5.5	45.41

[a]Values are mean ± SE (n = 6).

encapsulated in the microspheres was determined using the formula:

$$E = Q_p/Q_t \times 100$$

where E is the percentage encapsulation of microspheres, Qp is the quantity of drug encapsulated in microspheres (g), Qt is the quantity of drug added for encapsulation (g).

Particle size analysis

The particle size of microspheres was measured by optical microscopy. The microspheres were dispersed in water and a smear was made on a glass slide, and the size of 300 particles was measured by using a micrometer attached (Saravanan et al., 2008b) with a microscope. The particle size distribution of microspheres was plotted, and volume–surface mean diameter was determined using (Patrick, 2006) the following equation:

$$d_{vs} = \sum nd^3 / \sum nd^2$$

where d is the diameter of particle and n is the number of particles present in diameter d.

SEM

The sample for the SEM analysis was prepared by sprinkling the microspheres on to one side of double-adhesive stub. The stub was then coated with gold using Jeol JFC 1100 sputter coater (Jeol Ltd, Tokyo, Japan). The SEM analysis of the microspheres was carried out by using Jeol JSM 5300 (Jeol Ltd). The microspheres were viewed at an accelerating voltage of 15–20 kV.

FT-IR

Infrared spectra of diclofenac sodium and gelatin microspheres were taken by using KBr pellet technique and were recorded on Bomem MB-II FT-IR spectrometer, Quebec, Canada.

DSC

DSC of diclofenac sodium and microspheres was performed using PerkinElmer DSC7 model, Waltham, MA, USA. The instrument was calibrated with indium. All the samples (~5 mg) were heated in aluminum pans using dry nitrogen as the effluent gas. The analysis was performed with a heating range of 50–350°C and at a rate of 20°C/min.

XRD

Diclofenac sodium and microspheres were subjected to XRD study in an X-ray diffractometer (XD-D1; Shimadzu), within the range 5–70° of 2θ. The working conditions were CuKα radiation, 30 kV, 20 mA and with a slit of 1-1-0.3 mm.

Gas chromatography to test residual glutaraldehyde

One gram of microspheres, from each of the different batches, was extracted with 25 ml of water by shaking occasionally for 24 h. Then, the extract was filtered and 2 μl of filtrate was injected into gas chromatographic system for glutaraldehyde determination as explained in our previous work (Saravanan et al., 2008b).

In vitro *release of diclofenac sodium*

The *in vitro* release studies (Saravanan et al., 2004) of drug-loaded microspheres were carried out at 37°C in the phosphate buffer (pH 7.4). Each batch of microspheres, equivalent to 20 mg of diclofenac sodium, was individually added to 100 ml of the phosphate buffer (pH 7.4) in flasks. The flasks were shaken (60 oscillations/min) in an incubator (Remi) at 37°C. One milliliter of sample was withdrawn at regular time intervals and the same volume of phosphate buffer was replaced. After suitable dilution, diclofenac sodium content in phosphate buffer (pH 7.4) was estimated at 277 nm using a UV visible spectrophotometer (Shimadzu 1601).

Release kinetics

Data obtained from *in vitro* release studies were fitted to various kinetic (Costa and Sousa Lobo, 2001; Saravanan et al., 2004) equations. The kinetic models used are zero-order, first-order, and Higuchi equations. The following plots were made: Q_t versus t (zero-order kinetic model); log ($Q_0 - Q_t$) versus t (first-order kinetic model); Q_t versus square root of t (Higuchi model) where Q_t is the amount of diclofenac sodium released at a time t and Q_0 is the initial amount of the diclofenac sodium present in microspheres. Further, to find out the mechanism of drug release, first 60% drug release was fitted to the Korsmeyer–Peppas model:

$$M_t/M_\alpha = kt^n$$

where M_t/M_α is the fraction of drug released at a time t, k is the rate constant, and n is release exponent. The n value is used to characterize different release mechanisms.

Determination of targeting efficiency

The percentage of injected drug available at the site of action was determined in normal rabbits ($n = 6$) as follows. The institutional ethical committee for animal experimentation, Vel's College of Pharmacy, Chennai, India approved all experimental procedures. The gelatin microspheres (batch no: GM_2) equivalent to 10 mg of drug were dispersed in 0.5 ml of normal saline and

sonicated for 2 min to get uniform suspension. Then, the contents were transferred to a 1 ml syringe with 27-gauge needle and injected into the left knee joint (target site). All injections were done under anesthesia (phenobarbitone 15–25 mg/kg, intraperitoneal) in order to avoid the pain and stress to animals.

After 1 and 7 days of postinjection, animals (n =3) were sacrificed (intraperitoneal injection of phenobarbitone, 45–55 mg/kg) and the drug present in the target area was analyzed. The content of the synovium that has received an intra-articular injection of gelatin microspheres loaded with diclofenac sodium was transferred to 50 ml of 1N sodium hydroxide. The microspheres present in the synovial content were allowed to digest by the sodium hydroxide at room temperature for 12 h. After the digestion process, the contents were filtered to remove suspended matters. 5 ml of filtrate was taken in a test tube and shaken well with 5 ml of methanol for 30 min. The solution was centrifuged (3000 rpm; Remi) and 20 µl of clear supernatant liquid was injected into HPLC, Shimadzu, VP series to estimate diclofenac sodium as explained in our previous work (Saravanan et al., 2004).

Results and discussion

Preparation of microspheres

The microencapsulation process adopted in the study was good as indicated by the high yield of microspheres in Table 1. Higher yield reflects low wastage and high efficiency in the encapsulation process. The formulated microspheres were free flowing in nature. The usage of sesame oil with span 80 was found to be effective in dispersing aqueous globules containing drug and gelatin. The stirring conditions were optimized to get the required size (below 60 µm) by observing the globule size under the microscope. The temperature was maintained at 60°C until the completion of emulsification to facilitate microsphere formation. At 60°C, the gelatin solution is pourable and diclofenac sodium is soluble at specified concentrations as shown in Table 1. At the end of preparation, sodium metabisulphite was added to neutralize unreacted glutaraldehyde and the cross-linking was terminated (Öner and Groves, 1993).

Drug loading, entrapment and encapsulation efficiency

Sodium hydroxide solution was used to digest the gelatin in order to extract the encapsulated diclofenac sodium. Because the drug is soluble in sodium hydroxide, it is possible to get complete extraction of drug from the microspheres (Saravanan et al., 2004). Gelatin microspheres, based on the drug–polymer ratio, have shown 81–91% of entrapment and encapsulation efficiency as given in Table 1. During the cross-linking process, the gelatin microspheres will shrink and expel the drug molecules along with the water into the oil phase. This could be the reason for the loss of 10–20% of the drug during the encapsulation process. Moreover, higher drug loading lowered the percentage of entrapment and encapsulation, which indicates the wastage of drug during the microencapsulation process. This may be due to the drug present in the surface of microspheres with higher drug loading, which might be removed during washing and thus reducing the % of entrapment and encapsulation at higher drug loading.

Particle size analysis

In our series of work (Saravanan et al., 2003; 2004; 2008a; 2008b), we have formulated gelatin microspheres with different drug loading, gelatin content, stirring speed, and with varying degree of cross-linking. Microspheres loaded with high % of drug showed a faster release than the microspheres with lower drug loading and vice versa. The percentage of gelatin solution used to make microspheres also influences the particle size and *in vitro* release. Gelatin solution of 25% w/v or more are required to make microspheres in order to get the drug release for more than 30 days. As viscosity is high for a solution prepared with 20–25% w/v of gelatin, high stirring speed is required to get smaller microspheres. Based on our experience, we have selected suitable formulation conditions such as drug/gelatin ratio, volume of internal phase, external phase, addition of surfactant and stirring speed to get a particle size range of 10–60 micrometer. Smaller microspheres, less than 5 microns in size, were rapidly cleared from the joint after intra-articular administration. Moreover, smaller particles will have less sustained drug release than larger size particles due to a difference in surface area. At the same time, the particle size should be within the range that can be injected using a conventional needle and should be accommodated within the synovium. Particles in the range of 1–70 µm have been employed in intra-articular delivery and larger size particles have shown better retention time/sustained effect (Larsen et al., 2008). Based on this information, we have attempted to formulate gelatin microspheres in the range of 20–70 µm to get maximum retention at the injected joint and prolonged drug release.

The size distribution of formulated gelatin microsphere loaded with diclofenac sodium for intra-articular injection (Figure 1) was between 1 and 60 µm with an average size of 31–45 µm. The average size of unloaded gelatin microspheres was smaller than the drug-loaded microspheres. The average size of gelatin microspheres with 0, 9.8, 18.3, 26.7, and 33.1% of diclofenac sodium were 31.01, 37.89, 40.62, and 45.41 µm, respectively. The particle size distribution of drug-loaded microspheres was more or less the same irrespective of drug content. Increase in

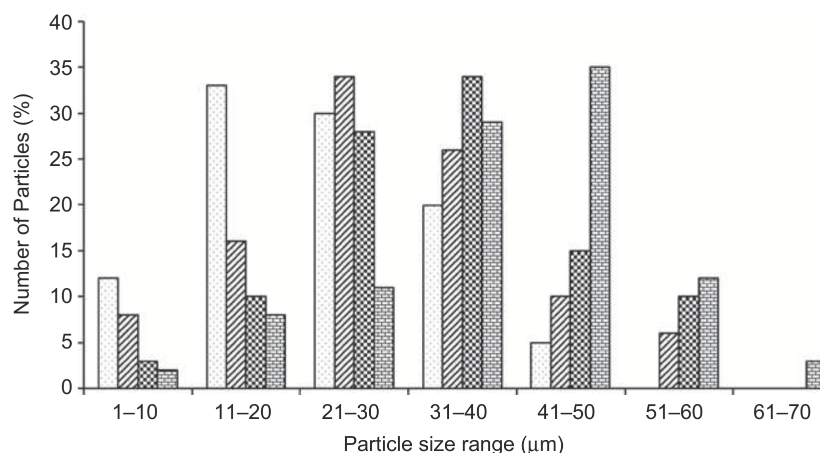

Figure 1. Particle size distribution of gelatin microspheres loaded with , 9.8, 18.3, and 26.7 % w/w of diclofenac sodium.

drug loading produced larger particles that resulted in an increase in the average particle size. All formulated microspheres were well below the size limit that can be injected intra-articularly using a conventional needle.

SEM

The compact nature and spherical shape of the gelatin microspheres were confirmed (Figure 2) by SEM. The particle size of the formulated gelatin microspheres was less than 50 μm as evidenced by the SEM photographs (Figure 2A and 2D). The surface of gelatin microspheres was smooth and showed no surface drug or particles (Figure 2B, 2C, 2E, and 2F). The gelatin microspheres showed only a little aggregation.

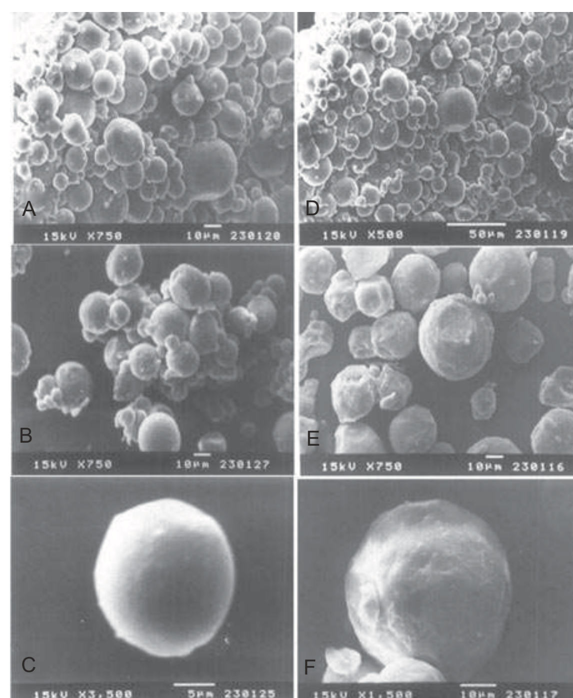

Figure 2. SEM photographs of (A–C) unloaded gelatin microspheres and (D–F) diclofenac sodium–loaded gelatin microspheres. A and D show the particle size distribution of unloaded and loaded microspheres, respectively. B and E show spherical geometry of formulated microspheres. C and F show the surface characters of unloaded and loaded gelatin microspheres, respectively.

FT-IR spectroscopy

The IR spectrum of unloaded gelatin microsphere is shown in Figure 3, a. The IR spectrum of diclofenac sodium (Figure 3, b) showed characteristic peaks at 1167 and 682 cm^{-1} for aromatic –C-Cl. The stretches between 800 and 600 cm^{-1} also support the presence of –C-Cl group. The strong peaks in the region of 1600–1700–1800 cm^{-1} indicate the presence of -C=O. Moreover, the presence of peaks in the region of 1250, 1283, and 1044 cm^{-1} confirms the –C-O- group. The peaks at 1603, 1507, and 869–716 cm^{-1} confirm the presence of an aromatic ring. The peaks of diclofenac sodium–loaded gelatin microspheres (Figure 3, c) were similar (but with lesser intensity) to the spectrum of diclofenac sodium. The peaks of various functional groups as described in the IR spectrum of diclofenac sodium were also present in the gelatin microspheres loaded with diclofenac sodium (Figure 3) without any shift or change. These observations revealed the intact nature of the diclofenac sodium present in the gelatin microspheres. From these results, the absence of drug–polymer interaction and the stability of the encapsulated drug in the gelatin microspheres were confirmed.

DSC

The thermogram of the diclofenac sodium and physical mixture of diclofenac sodium/unloaded gelatin

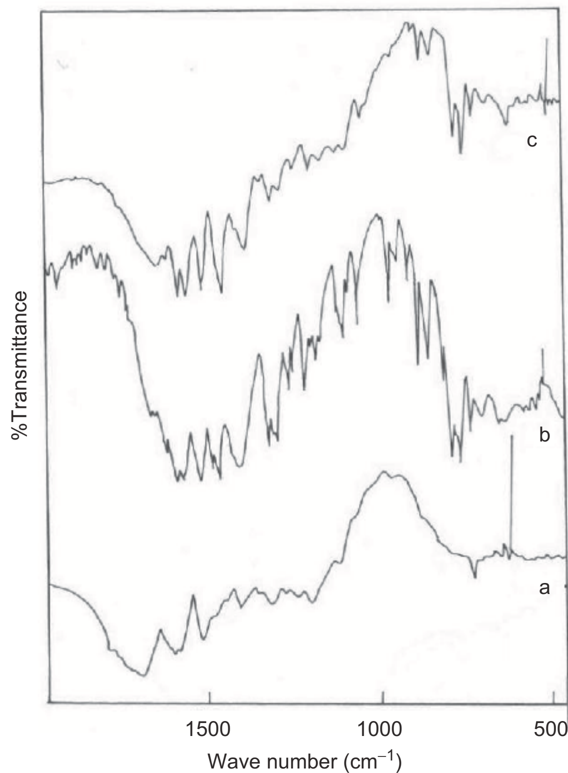

Figure 3. Fourier transform infrared spectrum of unloaded gelatin microspheres (a), diclofenac sodium (b), and gelatin microspheres loaded with diclofenac sodium (c).

microspheres showed (Figure 4, a and b) a peak at 297°C that is the melting point of diclofenac sodium. The thermograms of gelatin microspheres loaded with diclofenac sodium showed no peak (Figure 4, c) at 297°C and indicated amorphous nature of diclofenac sodium in the formulated microspheres. We have previously published similar effect observed with diclofenac sodium–loaded gelatin magnetic microspheres (Saravanan et al., 2004).

XRD

XRD patterns of gelatin microspheres without drug, diclofenac sodium, and diclofenac sodium–loaded gelatin microspheres are shown in Figure 5, a–c, respectively. XRD pattern of diclofenac sodium produced characteristic peaks as shown in Figure 5, b. These peaks are much less intense and disappeared in the XRD of drug-loaded gelatin microspheres (Figure 5, c), suggesting the presence of drug as molecular dispersion in the gelatin microspheres. This, in support with the DSC study, clearly reveals the amorphous form of entrapped drug in the microspheres.

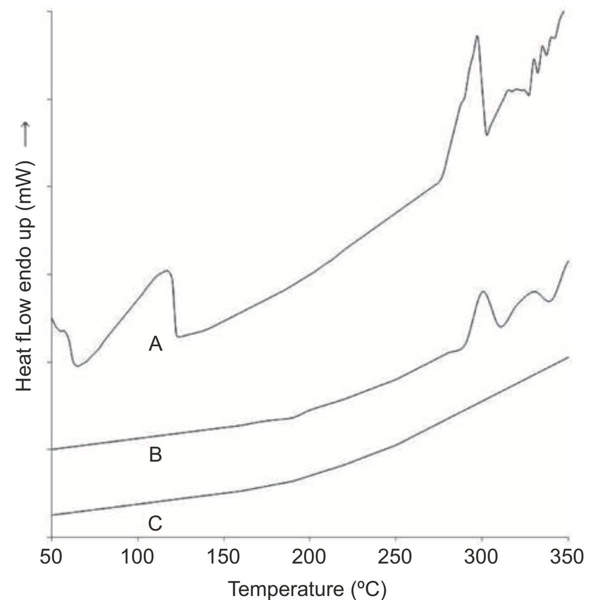

Figure 4. Differential scanning calorimetry of diclofenac sodium, (A) physical mixture of diclofenac sodium and unloaded gelatin microspheres (1:1) (B) and gelatin microspheres loaded with diclofenac sodium (C).

Test for absence of glutaraldehyde

The formulated microspheres must be free from residual glutaraldehyde, which is toxic and produce irritation/inflammation at the site of injection. To confirm its absence, the microspheres were tested for glutaraldehyde residue by gas chromatography. Sodium metabisulphite was used to remove the unreacted glutaraldehyde from the microspheres. All formulated microspheres were found to be free from residual glutaraldehyde.

Diclofenac sodium release from gelatin microspheres

The *in vitro* release of diclofenac sodium from formulated microspheres was tested in the phosphate buffer (pH 7.4). The *in vitro* release of drug from gelatin microspheres loaded with 9.8, 18.3, and 26.7% w/w diclofenac sodium was presented in Figure 6. In the phosphate buffer, the release was slow and extended for 30 days depending on the drug/gelatin ratio. The release was biphasic with an initial burst effect, which may be useful to produce an immediate therapeutic effect; later, the release became very slow and prolonged. The release rate of drug was influenced by the drug loading as shown in Figure 6. Gelatin microspheres prepared with 9.8% w/w of drug loading released the drug very slowly in the phosphate buffer (pH 7.4), whereas the microspheres prepared with higher drug loading (18.3 and 26.7% w/w) released the drug at a higher rate.

Release kinetics

The data obtained from the first 90% release were fitted to various kinetic equations to determine the mechanism of drug release and release rate. As indicated by the higher correlation coefficient (r^2), the drug release from gelatin microspheres (Table 2) followed the Higuchi model rather than the first-order and zero-order equations. These findings indicated that the drug release from the formulated gelatin microspheres was diffusion controlled. To confirm the release mechanism, the data from the first 60% drug release were applied to the Korsmeyer–Peppas equation to find out the release exponent n that indicates the mechanism of drug diffusion. The data were well fitted with the equation as indicated by the high correlation coefficient (r^2) and the n values were between 0.27 and 0.5 indicating the Fickian model. This confirms the drug-release mechanism as diffusion as it adheres to Fick's law (Patrick, 2006), and this prediction is further supported by high correlation obtained in the square root equation (Higuchi model).

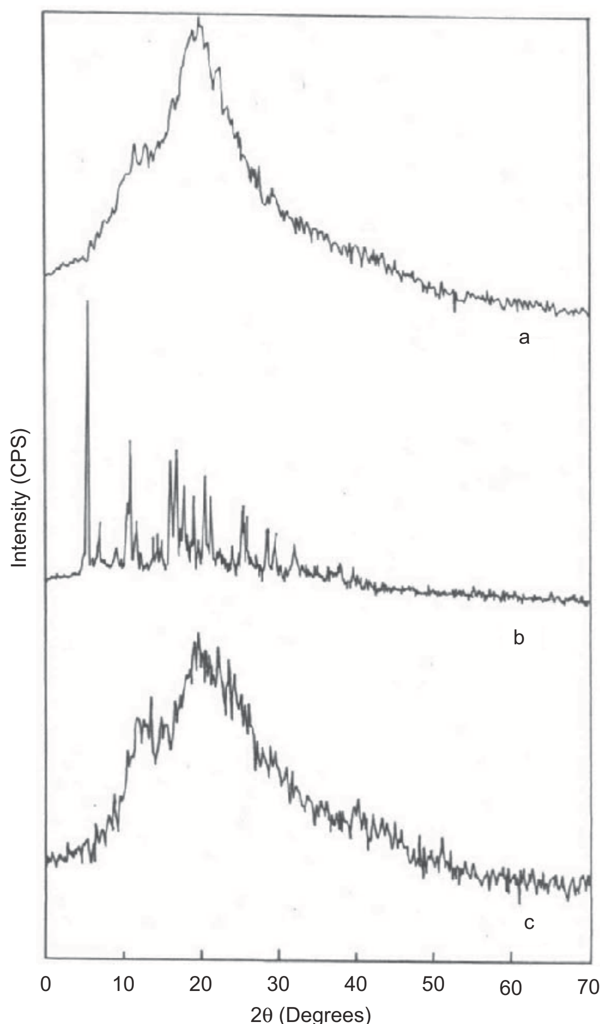

Figure 5. X-ray diffraction patterns of unloaded gelatin microspheres (a), diclofenac sodium (b), and 26.7% w/w drug-loaded gelatin microspheres (c).

In vivo *studies*

About 81.2 ± 7.4 and 43.7 ± 9.1% of administered drug in the microspheres were recovered from the target joint after 1 and 7 days of postinjection, respectively. The remaining might be released/distributed/excreted

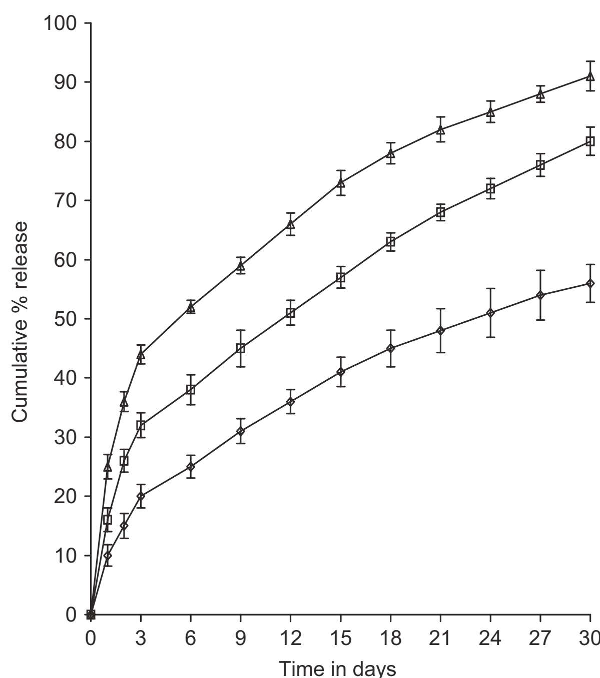

Figure 6. The in vitro release of diclofenac sodium from gelatin microspheres with 9.8 (-◇-), 18.3 (-□-), and 26.7 (-△-) % w/w drug loading in the phosphate buffer (pH 7.4). Values represent a mean of six determinations and bars represent ± SE.

Table 2. Square parameters of the model equations applied to the release of diclofenac sodium from gelatin microspheres formulated for intra-articular administration in the phosphate buffer pH 7.4.

	Zero-order equation		First-order equation		Higuchi model		Korsmeyer–Peppas	
Batch no.	r^2	k_0 (d^{-1})	r^2	k_1 (d^{-1})	r^2	k_H ($d^{-1/2}$)	r^2	n
GM1	0.9289	1.67	0.9742	0.0256	0.9975	10.30	0.9960	0.50
GM2	0.9158	2.27	0.9871	0.0479	0.9932	14.01	0.9730	0.43
GM3	0.8462	2.63	0.9827	0.0707	0.9747	15.92	0.9686	0.38

GM1, GM2, and GM3 indicate gelatin microspheres loaded with 9.8, 18.3, and 26.7% w/w of diclofenac sodium, respectively.

during the time period between the administration and estimation of drug. The formulated microspheres showed higher percentage (81.2%) of targeting than the gelatin magnetic microspheres administered via intravenous (1.1%) and intra-arterial (70.7%) route as reported (Saravanan et al., 2004; 2008b) in our previous study. This could be due to the size of the particles employed in the study that is trapped inside the synovium after the injection. As the majority of particles are larger than 10 μm, upon intra-articular injection, the chance of entering into systemic circulations becomes highly unlikely and therefore, the particles are retained in the joint for more than a week. Apart from intra-articular administration, the formulated microspheres can be injected intramuscularly to form a depot, from which the drug will be released for a prolonged period, which could be useful in polyarthritis where multiple joints are affected.

Conclusion

Diclofenac sodium–loaded gelatin microspheres were formulated for intra-articular administration with acceptable physicochemical properties. The microspheres showed spherical geometry with fewer aggregations. The amorphous nature of entrapped diclofenac sodium in gelatin microsphere was confirmed by the DSC and XRD studies. The formulated microspheres could prolong the drug release up to 30 days. *In vivo* study revealed the capability of formulated gelatin microspheres to be localized in the target area. Further *in vivo* studies are required with a greater number of animals to find out the feasibility for clinical application.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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