In vitro and in vivo evaluation of diclofenac sodium loaded albumin microspheres

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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. Among the microparticulate systems, microspheres have a special importance since it is possible to target drugs and provide controlled release. Diclofenac sodium (DS), is a potent drug in the NSAID group having non-steroidal, anti-inflammatory properties, and is widely used in the treatment of rheumatoid arthritis, osteoarthritis and ankylosing spondylitis. In this present study, it was aimed to prepare microsphere formulations of DS using a natural biodegradable polymer as a carrier for intraarticular administration to extend the duration period of the dosage form in the knee joint. Microsphere formulations of DS which were prepared were evaluated in vitro for particle size, yield value, encapsulation efficiency, surface morphology, and in vitro drug release. Two appropriate formulations were selected for in vivo trials. For the in vivo studies, Technetium-99m labelled polyclonal human immunogammaglobulin (99mTc-HIG) was used as the radiopharmaceutical to demonstrate arthritic lesions by gamma scintigraphy. After the induction of arthritis in knee joints of rabbits, the radio-labelled microspheres loaded with DS were injected directly into the articular cavity and at specific time points gamma scintigrams were obtained to find the residence time of the microspheres in knee joints in order to determine the most suitable formulation.

Keywords: Diclofenac sodium, microspheres, in vitro, arthritis, scintigraphic imaging.

Introduction

The first biodegradable and biocompatible uniformly sized albumin microspheres were formulated in the 1970s (Kramer 1974). Initially, microspheres were considered as drug carrier systems having a diagnostic goal, later their importance as drug delivery systems was well understood and documented. Among the various polymeric carrier systems, albumin microspheres have been shown to be very suitable systems for drug targeting and drug delivery, due to their advantages in biodegradability, lack of toxicity and antigenicity (Schafer et al. 1994, Muller et al. 1996). Furthermore, albumin microspheres are stable from the physical and

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chemical aspect, and have the capability to be removed from the vascular system by phagocytosis (Egbaria and Friedman 1990).

Albumin microspheres were first described by Kramer (1974) as the best tolerated drug delivery systems by synovial tissue when compared to biodegradable polymers such as poly(lactide), poly(butylycycanoacrylate) and gelatin (Ratcliffe et al. 1987, Gupta and Hung 1989, Bogdansky 1990). They are metabolized in the body, and the factors influencing the extent of metabolism can be stated as the size of particles, degree of stabilization and the site of metabolism (Bernard et al. 1980). Drug release from microspheres can be widely modulated by the extent and nature of cross-linking, size, the position of the drug and its incorporation level in the microspheres (Tomlinson et al. 1984).

The administration of NSAIDs into the intra-articular cavity in patients with chronic inflammatory disease is complicated due to the short duration of effect. The principal requirement for an ideal intra-articular drug carrier system is to retain the active substance in the knee joint until it is taken up by the phagocytic cells. Incorporation of drugs within biodegradable polymer particles proved to be effective in improving the retention of drug within the joint cavity.

Diclofenac sodium (DS) is a sodium salt of an aminophenyl acetic acid which is rapidly absorbed after oral administration, but following the administration by this route, common side effects are observed, such as gastritis, peptic ulcer and bleeding. Among the NSAIDs, DS appears to be one of the most effective anti-inflammatory drugs in the treatment of rheumatoid arthritis, osteoarthritis, and ankylosing spondylitis.

The objective of this study was to prepare biodegradable microspheres of DS using a natural polymer, bovine serum albumin, as the drug carrier for intra-articular administration, in order to circumvent the above stated side effects, to evaluate the properties of biodegradable microspheres in vitro, and afterwards to investigate the retention of DS microspheres in inflamed knee joints of rabbits. DS incorporated microspheres of bovine serum albumin (BSA) were prepared by the emulsion polymerization method (Tu̇ncay et al. 1997, 1998).

Materials and methods

Materials

The active substance was diclofenac sodium (DS) (Novartis, Switzerland), the matrix material was BSA (Armour Pharmaceutical Company, USA) and the hardening agent used was glutaraldehyde (25% aqueous solution, Merck, Germany). Technetium-99m as pertechnetate was obtained from a generator (Amersham International, England). Polyclonal human immunoglobulin kits were obtained from Mallincrot (Holland). Ovalbumin and Freund's complete adjuvant were bought from Sigma (USA). White female New Zealand rabbits were raised locally.

Methods

Preparation of microspheres. A previously used procedure (Vural et al. 1990) was followed with some modifications. The schematic representation of the preparation is shown in figure 1. In the preparation of DS loaded albumin microspheres
by the emulsion polymerization method, different duration times (15, 60 min) and various concentrations of the stabilizing agent (0.1, 0.7 ml glutaraldehyde of 25% aqueous solution) were used (table 1). Briefly, 0.5 ml of the aqueous solution, containing 25% BSA (w/v) and DS were mixed with 100 ml of cottonseed oil at 1400 rpm and 25°C. The BSA microspheres were washed with diethyl ether (anhydrous) to remove the oil phase. The resulting microspheres were dried in a vacuum oven overnight and stored at 4°C.

**Physicochemical characteristics of microspheres**

- **Determination of DS content of microspheres.** 10 ml 0.5N NaOH: 70% Ethanol (1:1) solution was added to 10 mg accurately weighed DS microspheres. This mixture was kept in an ultrasonic bath until the DS was completely
Table 1. Formulation of DS containing BSA microspheres.

<table>
<thead>
<tr>
<th>Code</th>
<th>Duration of stabilization (min)</th>
<th>Concentration of the cross-linking agent (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>60</td>
<td>0.7</td>
</tr>
<tr>
<td>B</td>
<td>60</td>
<td>0.1</td>
</tr>
<tr>
<td>C</td>
<td>15</td>
<td>0.7</td>
</tr>
<tr>
<td>D</td>
<td>15</td>
<td>0.1</td>
</tr>
</tbody>
</table>

dispersed. After centrifugation, the supernatant was decanted and assayed at 283 nm spectrophotometrically to determine the amount of drug present at the surface of microspheres. The residue was dissolved in methanol after drying, and the absorbance was read at 283 nm by a spectrophotometer (Shimadzu UV-160 A). By this method, the amount of entrapped drug was calculated.

- **Surface morphology.** The surface characteristics of the microspheres were studied with a scanning electron microscope (Jeol-SEM ASID-10 Device in 80 KV). Microspheres were mounted on metal stubs with conductive silver paint and then sputtered with a 150 A thick layer of gold in a BIO-RAD apparatus.

- **Particle size measurements.** The particle size was determined by means of a laser diffractometer (HELOS, Sympatec). The microspheres were suspended in distilled water and blended in 0.1% Tween 80. The particle size was measured after stirring and ultrasonification for 5 min.

- **In vitro release studies.** Dissolution tests in phosphate buffer (pH 6.8) were conducted to obtain DS release profiles. An accurately weighed amount of microspheres (50 mg) containing DS were suspended in buffer solution (25ml) at constant temperature (37±0.5°C) in a horizontal shaker (50cpm). The suspension was shaken continuously and, at various time periods (0.25-8 h), 1 ml of the sample solutions was withdrawn and an equivalent volume of fresh buffer was added into the dissolution medium.

**In vivo studies**

Mono-articular arthritis was induced in the left knee joints of white female New Zealand rabbits \((n = 15)\) weighing approximately 3 kg, by modification of the method applied by Ratcliffe et al. (1984). Ovalbumin was used as the antigen and was emulsified with Freund's Complete Adjuvant. A physiological solution (0.9% NaCl) of albumin at a concentration of 20mg/ml was emulsified with an equal volume of Freund's Complete Adjuvant. In order to obtain an homogeneous dispersion, the emulsion was passed through a homogenizer. 1 ml of the emulsion sample was injected into the left knee joint of each rabbit. The contralateral knees were used as the control joints.

Technetium-99m labelled polyclonal human immunogamma-globulin \((^{99m}Tc-HIG)\) was used as the radiopharmaceutical to demonstrate arthritis lesions by gamma scintigraphy before therapy (Rubin et al. 1989, Nasirideen et al. 1998). Four days after the induction of arthritis, each rabbit was administered with 1 mCi (0.2ml) of \(^{99m}Tc\)-HIG intravenously through the ear vein in saline solution. Four hours post-administration of the radiopharmaceutical static images were obtained
Table 2. Characterization of BSA microspheres.

<table>
<thead>
<tr>
<th>Code</th>
<th>Yield value (%)</th>
<th>Surface drug content (%)</th>
<th>Entrapped drug content (%)</th>
<th>Total drug content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>40.18</td>
<td>6.80</td>
<td>4.46</td>
<td>11.26</td>
</tr>
<tr>
<td>B</td>
<td>51.70</td>
<td>9.28</td>
<td>2.55</td>
<td>11.83</td>
</tr>
<tr>
<td>C</td>
<td>42.00</td>
<td>12.69</td>
<td>2.20</td>
<td>14.89</td>
</tr>
<tr>
<td>D</td>
<td>52.65</td>
<td>9.93</td>
<td>2.57</td>
<td>12.50</td>
</tr>
</tbody>
</table>

from the posterior position with a gamma camera using a LEAP collimator; 300—590 kilocounts were collected to obtain an image. Regions of interest were drawn over both arthritic (target) and contralateral (non-target) knee joints. The radioactive count ratios obtained from the equal regions of interest of arthritic (A) and the control areas (C) were used to determine the A/C ratios.

The labelling efficiency of $^{99m}$Tc labelled HIG was determined by impregnated thin-layer chromatography (ITLC), using ITLC-SG mini strips and saline solvent (Saha 1992). DS containing microspheres were used as the test sample. Blank microspheres and DS solution in buffered saline were used as control samples. Samples were administered to rabbits intra-articularly one day after demonstration of arthritis lesions. DS containing preparations were administered at a dose of 2mg/0.5ml to each rabbit in their respective groups. Blank microspheres and DS solution in buffered saline were administered, as stated above (0.5ml), to each rabbit in the respective group.

Evaluation of arthritis was performed by the i.v. injection of 1 mCi of $^{99m}$Tc-HIG as explained before on days 3, 17 and 30. Four hours post-administration of the radiopharmaceutical, scintigraphic images of rabbits were obtained, as described previously. Regions of interest were created in the target and non-target areas and the radioactivity count ratios (A/C) in each group were determined. The average values of each group, with their standard deviations are presented in table 3.

### Results and discussion

In this study, the active substance was administered intra-articularly in the form of microspheres in a biocompatible and biodegradable matrix system to extend the retention of DS within the joint cavity, thus improving therapy and

Table 3. Measurements of average radioactivity count ratios (Mean ± Sd) at 4 h in ratios of target to non-target areas before and after therapy. (A) Rabbit treated with DS containing BSA microspheres, (B) Rabbit treated with blank BSA microspheres, and, (C) Rabbit treated with DS in saline solution.

<table>
<thead>
<tr>
<th>Code</th>
<th>n</th>
<th>Pre-treated ± SD</th>
<th>Post-treated ± SD (3rd day)</th>
<th>Pre-treated ± SD (17th day)</th>
<th>Pre-treated ± SD (30th day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5</td>
<td>2.320 ± 0.710</td>
<td>2.110 ± 0.977</td>
<td>3.212 ± 1.357</td>
<td>1.472 ± 0.567</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>2.295 ± 0.178</td>
<td>2.568 ± 0.791</td>
<td>2.190 ± 1.020</td>
<td>2.933 ± 0.425</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>2.233 ± 0.401</td>
<td>2.296 ± 0.423</td>
<td>2.442 ± 0.979</td>
<td>1.742 ± 0.723</td>
</tr>
</tbody>
</table>
minimizing the adverse effects of the antiinflammatory drug. BSA was selected as a natural matrix material for its non-antigenic property and ability to control the physicochemical characteristics of the microspheres produced, depending on the cross-linking methods and characteristics of cross-linking agent. Since bovine serum albumin and the active substance diclofenac sodium were water-soluble, the emulsion polymerization method was selected as the preparation procedure for the microspheres. In this preparation method, it was critical to use anhydrous ether to prevent the dissolution of DS by water which might be present in ether, thus increasing the loading efficiency of the drug and the yield of microspheres.

In order to evaluate the influence of the concentration of the cross-linking agent and duration of stabilization on the characteristics of microspheres, glutaraldehyde was used in two different concentrations (0.7, 0.1 ml) and stabilization was performed using two different duration times (15, 60min).

The particle size of each batch was measured individually and the mean diameter of the microspheres was determined to be approximately 15 µm. Light and scanning electron microscopy studies revealed that the albumin microspheres were spherical in shape, had a smooth surface and a uniform size distribution. There were no drug crystals present on the surface (figure 2). Other characterization data concerning the albumin microspheres are shown in table 2. The yield of the microspheres was in the range of 40—50%. It was observed that an increase in cross-linking agent and cross-linking degree caused a decrease in the yield; similar results were obtained by a previous researcher (Vural et al. 1990).

When determining loading efficiency, the fact that DS might be present both on the surface and entrapped in microspheres was taken into consideration. Thus, it was concluded that there was a variation in loading efficiency related to the

Figure 2. (a) Surface morphology of DS containing BSA microspheres. Light microscopy photograph.
Figure 2. (b) Surface morphology of DS containing BSA microspheres. Scanning electron microscopy (SEM) photograph (before the \textit{in vitro} release).

Figure 2. (c) Surface morphology of DS containing BSA microspheres. Scanning electron microscopy (SEM) photograph (after the \textit{in vitro} release).
concentration of cross-linking agent and duration of stabilization. The decrease in loading efficiency for microspheres which were formulated with 0.7 ml of glutaraldehyde (25%) and 60 min of stabilization time can be considered as an indication of this effect. Furthermore, when the percentage of drug present on the surface of microspheres was calculated, an increase, as the amount of stabilization agent and stabilization time increased, was observed (6.80% in A, 9.93% in D formulation). Another point that should be emphasized was the difficulty encountered in extracting the entrapped active substance DS from albumin microspheres in formulation A.

The drug release from microspheres is dependent on the drug (position of drug in the microsphere, molecular weight, concentration of the drug), the microspheres (size and density of microspheres, the extent and nature of cross-linking), and the release medium (pH, polarity) characteristics (Tomlinson et al. 1984). In this study, the effects of different cross-linking processes, concentrations of stabilization agent and duration of stabilization were investigated on drug release properties.

The kinetics of release of DS from BSA microspheres showed a biphasic release pattern with an initial release (burst effect) followed by a slower release. The burst effect could be attributed to the presence of drug crystals spread over the periphery of the DS microspheres.

The release in D formulation in which the concentration of stabilization agent was 0.1 ml and stabilization time was 15 min was much faster than the B formulation with a cross-linking period of 60 min. When A and D formulations were compared in which the concentration of cross-linking agent was 0.7 and 0.1 ml and duration of stabilization was 60 and 15 min, respectively, it was observed that A formulation released 90.4% of the active substance in 7 h, whereas D formulation released 98.8%, immediately in 30 min (figure 3). The results indicated that, as the stabilization degree increased, the time for drug release was extended (Gupta and Hung 1989, Gupta et al. 1989, Tunçay et al. 1997, Çalış et al. 1998).

Figure 3. In vitro release profiles of DS microspheres.
For the evaluation of the in vivo studies, regions of interest (ROIs) were drawn, both in the arthritic and normal contralateral knee joints, and radioactivity count ratios of target to non-target areas were calculated with standard deviations of mean values Tc-HIG demonstrated arthritic lesions very well (figure 4) and the labelling efficiency of the $^{99m}$Tc-HIG complex was determined as 99%.

No significant difference was found at pretreatment, 3rd and 17th days between the DS containing and blank, BSA microspheres. But, in respect to the data obtained at the 30th day, a significant difference emerged between DS containing and blank microspheres of BSA. A decrease has occurred in the radioactivity count ratios of the DS microsphere group and DS in saline solution group. Therefore, it
was concluded that the DS loaded BSA microspheres could be a more effective treatment. Similar results with natural polymers were obtained in the literature previously (Ratcliffe et al. 1984).

As a result of this study, it seems to be a good approach to prepare DS microspheres using the natural biodegradable polymer, bovine serum albumin, as a carrier matrix to provide an extended duration of active substance in the knee joint. Concentrations of stabilizing agent and duration of stabilization are determined to be critical parameters for release characteristics. Results of in vivo studies also seem promising.

References


