4. PREPARATION OF GELATIN MICROSPHERES

Intra-articular delivery of drug loaded microspheres has been developed by many authors (Ratcliffe et al., 1987; Pavanetto et al., 1994; Tuncay et al., 2000a; Bozdag et al., 2001) to target the drug in the arthritic joint and to get prolonged therapeutic effect. This microsphere system though delivered the drug in sustained manner but fails to regulate the release according to disease condition. In this study, we attempted to develop gelatin microspheres, which can regulate the drug release according to disease condition. As reported in literature, gelatin microspheres loaded with drug can be prepared by one of the following method.

A. **Emulsification and cross-linking method**: Aqueous solution of drug and gelatin were emulsified in oil. Then the dispersed aqueous phase containing drug and gelatin were hardened to produce microspheres by using cross-linking agents such as formaldehyde or glutaraldehyde (Tabata and Ikada, 1987; Leucuta, 1990; Lou et al., 1994; Narayani and Rao, 1994; Nastruzzi et al., 1994). Microspheres with required particle size, shape and drug release kinetics could be prepared by this method, and hence in the present study we have chosen this method for the preparation of gelatin microspheres.

B. **Coacervation/phase separation technique**: The drug to be encapsulated was suspended in the aqueous solution of gelatin. The coacervation was induced by adding incompatible solvent or polymer (Deasy, 1984; Saravanan et al., 2002). Then the coacervated gelatin droplets were used to encapsulate the suspended particles to form microcapsules.

C. **Congealable disperse phase encapsulation**: The drug and gelatin solution at higher temperature (40-60°C) was allowed to pass through a cooled column or drug-
gelatin solution is emulsified in oil and subsequently cooled (Li et al., 1997). This process will harden the gelatin solution-containing drug to form microparticles.

The drug release from the gelatin microsphere is mainly depend upon the rate of swelling in the dissolution medium which may be influenced by the following parameters (Öner and Groves, 1993; Ugwoke and Kinget, 1998).

- **Bloom strength**: Usage of higher bloom strength gelatin in microsphere formulation results in slower drug release.

- **Gelatin concentration and drug loading**: Gelatin microspheres with higher drug loading comparatively release the drug fastly. Usage of high concentration gelatin solution in the preparation of microspheres will yield microspheres of larger size and slower drug release.

- **Extent of cross-linking**: This is very crucial in the preparation of gelatin microspheres. The drug release property of gelatin microspheres can be modified to get required release by optimizing degree of cross-linking.

- **Particle size**: In general, in identical preparation conditions, larger particles will give prolonged release of the drug than the smaller particles.

In the present study, gelatin microspheres loaded with diclofenac sodium were prepared by emulsification and cross-linking method using glutaraldehyde as cross-linking agent.

### 4.1. Materials and Methods

Gelatin, type-B, 300 bloom strength was purchased from Sigma Chemicals, U.S.A. Bacterial collagenase, type-3 was obtained from CALBIOCHEM, California, U.S.A. Diclofenac sodium, gifted by 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.

4.1.1. Determination of diclofenac sodium: Preparation of calibration curve.

A standard solution of diclofenac sodium (1 mg/ml) in phosphate buffer (pH 7.4) was prepared with (100 μg/ml) and without collagenase. A suitable volume of solution was diluted to various concentrations ranging from 2.5 to 35 μg. These standard samples were analyzed at 277 nm using UV visible spectrophotometer (Shimadzu 1601).

4.1.2. Preparation of saturated solution of glutaraldehyde in toluene

Equal quantity of aqueous glutaraldehyde solution and toluene was taken (Yan et al., 1991) in a separating funnel and shaken for 1 hour to allow the saturation of glutaraldehyde in toluene. Then the aqueous phase and toluene phase was separated. Thus, obtained toluene saturated with glutaraldehyde was used to cross-link gelatin microspheres.

4.1.3. Preparation of gelatin microspheres loaded with diclofenac sodium

As given in the Table. 4.1, required quantity of gelatin was dissolved in 3 ml of phosphate buffer (pH 7.4) by heating to 60ºC. Specified quantity of diclofenac sodium was dissolved separately in 3 ml of phosphate buffer (pH 7.4) by heating and added to gelatin solution. Then the mixture was added drop wise to 100 ml of sesame oil with 1% w/v span 80 preheated to 60ºC and emulsified by stirring with the help of hand blender (5,000 rpm/3 min). Then the stabilized emulsion was stirred with the help of a stirrer attached to a motor (Remi, India, approx.1000 rpm). Required quantity of glutaraldehyde-saturated toluene solution (1, 5 and 10 ml) was added drop wise and the stirring was continued at room temperature for required time period (2, 4 and 6 h). The
cross-linked microspheres were collected by filtration by using Whatman filter paper (No: 41). After filtration, the microspheres were washed with anhydrous ether to remove sesame oil. Then it was washed with 3x10 ml of 5% w/v sodium metabisulphite, 2x10 ml water and 2x10 ml of isopropyl alcohol. After washing, the microspheres were dried at 45ºC, transferred to glass vials and stored in desiccator.

**Table 4.1. Formula and yield of gelatin microspheres**

<table>
<thead>
<tr>
<th>Batch No.</th>
<th>Amount of gelatin used (mg)</th>
<th>Amount of diclofenac sodium used (mg)</th>
<th>Yielda (mg)</th>
<th>Theoretical loading of diclofenac sodium (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM₀</td>
<td>1500</td>
<td>-</td>
<td>1466 ! 82</td>
<td>0</td>
</tr>
<tr>
<td>GM₁</td>
<td>1500</td>
<td>180</td>
<td>1630 ! 43</td>
<td>10.7</td>
</tr>
<tr>
<td>GM₂</td>
<td>1500</td>
<td>380</td>
<td>1770 ! 52</td>
<td>20.2</td>
</tr>
<tr>
<td>GM₃</td>
<td>1500</td>
<td>650</td>
<td>1980 ! 65</td>
<td>30.2</td>
</tr>
</tbody>
</table>

aValues are mean ! SE (n=6).

**4.1.4. Effect of proteolytic enzyme on formulated microspheres**

Gelatin microspheres prepared with various drug loading, extent and duration of cross-linking were separately added to 25 ml of phosphate buffer containing collagenase, in 100 ml conical flask. The flasks were kept in an incubator at 37ºC for 6 h and shaken occasionally. At different time intervals, one ml of sample was taken and analyzed for drug content by spectrophotometer at 277 nm. After each sampling, same quantity of fresh phosphate buffer was replaced.
4.2. Results and Discussion

Preparation of microspheres

The microencapsulation process adopted in the study produced good yield of microspheres with free flowing nature. The usage of sesame oil with span 80 was found to be effective in dispersing aqueous globules containing drug and gelatin and produced good yield of microspheres. The stirring conditions were optimized to get the required size (below 50 μm) by observing the globule size under the microscope. The cross-linking of gelatin microspheres were done by using glutaraldehyde-saturated toluene solution. At the end of preparation, by adding sodium metabisulphite, which was used to neutralize unreacted glutaraldehyde, the cross-linking was terminated (Öner and Groves, 1993).

The arthritis is a disease associated with involvement of proteolytic activity in the joint due to presence matrix metalloproteases (MMPs). MMPs are implicated in cartilage degradation in both rheumatoid arthritis and osteoarthritis. The two main families of MMPs believed to be responsible for cartilage degradation are collagenases and proteoglycanases (Testa et al., 1994; Patel et al., 1998). No data are available in the literature about the effect of proteolytic enzyme on the microspheres formulated for intra-articular administration. The presence of these enzymes may digest the microspheres made up of polymers such as albumin and gelatin, which consequently may leads to failure of therapy due to immediate release of loaded drug. In order to find the effect of proteolytic enzyme on the degradation of gelatin microspheres and to find out the suitable extent of cross-linking, proteolytic activity study was carried out as described in the experimental section. The quantity of drug released was observed as an indicator of microsphere degradation.
Determination of diclofenac sodium

The drug released by proteolytic degradation was estimated spectrophotometrically at 277 nm by using calibration curve (Figure 4.1, 4.2 and Table 4.2), prepared as explained in the experimental part.

Table 4.2. Absorbance (277 nm) of diclofenac sodium in phosphate buffer (pH 7.4) with and without collagenase.

<table>
<thead>
<tr>
<th>Standard No.</th>
<th>Concentration (μm/ml)</th>
<th>Absorbance</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2.</td>
<td>5</td>
<td>0.17</td>
<td>0.16</td>
<td>0.16</td>
</tr>
<tr>
<td>3.</td>
<td>10</td>
<td>0.33</td>
<td>0.31</td>
<td>0.31</td>
</tr>
<tr>
<td>4.</td>
<td>15</td>
<td>0.50</td>
<td>0.47</td>
<td>0.47</td>
</tr>
<tr>
<td>5.</td>
<td>20</td>
<td>0.66</td>
<td>0.62</td>
<td>0.62</td>
</tr>
<tr>
<td>6.</td>
<td>25</td>
<td>0.83</td>
<td>0.77</td>
<td>0.77</td>
</tr>
<tr>
<td>7.</td>
<td>30</td>
<td>1.02</td>
<td>0.93</td>
<td>0.93</td>
</tr>
</tbody>
</table>
Figure 4.1. Standard plot of diclofenac sodium in phosphate buffer (pH 7.4) with collagenase. The absorbance was taken at 277 nm.

$y = 0.0336x - 0.0032$

$R^2 = 0.9995$
Figure 4.2. Standard plot of diclofenac sodium in phosphate buffer (pH 7.4) without collagenase. The absorbance was taken at 277 nm.
The effect of drug loading on proteolytic degradation of gelatin microspheres

The effect of drug loading on proteolytic degradation of gelatin microspheres was shown in Figure 4.3. As the drug loading increased, the degradation increased. By the end of 6 h, at 100 μg/ml collagenase, the gelatin microspheres loaded with 30% drug released approximately 20% of the entrapped drug. This may be due to more surface area of gelatin microsphere, which resulted in faster dissolution of the poorly entrapped drug due to high loading, available for collagenase activity. Microspheres with 20% theoretical drug loading showed moderate release and was found to give optimum release rate. Microspheres loaded with 10% of drug showed little degradation with the given concentration of the collagenase. Based on these observations 20% drug loaded microspheres were selected to study the influence of duration crosslinking, quantity of glutaraldehyde added for crosslinking and concentration of collagenase on degradation of gelatin microspheres.

To find out the effect of duration of cross-linking on proteolytic degradation, gelatin microspheres with 20% theoretical drug loading was prepared with cross-linking duration of 2, 4, 6 h. As expected, longer duration of cross-linking resulted in slower drug release (Figure 4.4). Gelatin microspheres cross-liked with 10 ml of glutaraldehyde-saturated toluene for 6 h showed slower release rate and the microspheres prepared with below this cross-linking period were degraded fast hence may not be suitable for prolonged drug release. Based on these observations, it can be predicted that the gelatin microspheres prepared with cross-linking duration of above 6 h and 10 ml of glutaraldehyde-saturated toluene may release the drug more slowly, hence may not be suitable for effective therapy.
Figure 4.3. The effect of drug loading on proteolytic degradation of gelatin microspheres prepared by crosslinking with 10 ml of glutaraldehyde–saturated toluene for 6 h. The collagenase concentration used was 100 μg/ml. Values represent mean of six determinations and bars represent ± SE.
Figure 4.4. Effect of duration of cross-linking on proteolytic degradation of gelatin microspheres with 20% theoretical drug loading. The collagenase concentration used was 100 μg/ml. Values represent mean of six determinations and bars represent ± SE.
To find out the effect of quantity of glutaraldehyde added for crosslinking on proteolytic degradation, gelatin microspheres with 20% theoretical drug loading were prepared by cross-linking with 1, 5 and 10 ml of glutaraldehyde-saturated toluene. Higher extent of cross-linking considerably reduced the proteolytic degradation of gelatin microspheres. As shown in Figure 4.5, microspheres prepared with 1 ml of cross-linking agent released almost all the drug within 6 h, indicating complete degradation of microspheres. Microspheres prepared with 5 ml crosslinking showed moderate degradation, whereas microspheres prepared with 10 ml of cross-linking agent showed very slow degradation.

To study the effect of concentration of collagenase on microsphere degradation, gelatin microspheres loaded with 20% of drug and cross-linked with varying amount of glutaraldehyde-saturated toluene were observed for drug release, as an indicator for degradation, at the collagenase concentrations of 100 and 50 \( \mu \text{g/ml} \). The microsphere degradation with collagenase at 100 \( \mu \text{g/ml} \), as indicated in Figure 4.5, was comparatively fast and the microspheres prepared with 10 ml of glutaraldehyde-saturated toluene showed release up to 20%. As shown in Figure 4.6, the microspheres showed proteolytic resistance at the collagenase concentration of 50 \( \mu \text{g/ml} \) and the microspheres prepared with 10 ml of glutaraldehyde-saturated toluene released less than 2% of entrapped drug. The microspheres prepared with lesser quantity of cross-linking agents released the drug fast as shown in Figure 4.6.
Figure 4.5. Effect of quantity of glutaraldehyde added for crosslinking on proteolytic degradation of gelatin microspheres with 20% theoretical loading. The collagenase concentration used was 100 μg/ml. Values represent mean of six determinations and bars represent ± SE.
Figure 4.6. Effect of quantity of glutaraldehyde added for crosslinking on proteolytic degradation of gelatin microspheres with 20% theoretical loading. The collagenase concentration used was 50 μg/ml. Values represent mean of six determinations and bars represent ± SE.
From the Figures 4.3 to 4.6, it can be concluded that microspheres prepared by crosslinking with 10 ml of glutaraldehyde-saturated toluene for a duration of 6 h, seem to be optimum to withstand proteolytic enzymes and capable of releasing the drug for a prolonged period. The microspheres prepared with higher quantity of cross-linking agent and duration may be irrational, which might produce very slow release of the drug and result in ineffective therapy. Hence, gelatin microspheres prepared as per the formula given in Table 4.1, by using 10 ml of glutaraldehyde-saturated toluene solution and cross-linking duration of 6 h were selected for further studies.