Chapter 5

CHARACTERIZATION OF THE FORMULATIONS

5.1 Introduction

Characterization is a very important step in the formulation development. The in-vitro tests are carried out to predict the in-vivo behavior of the formulation. For its optimum performance in-vivo, the formulation should possess specific characteristics. Thus in the in-vitro analysis of the formulation, it is checked whether the formulation has optimum characteristics to give the desired results in-vivo. In-vitro tests are very helpful to compare the formulation having different compositions and choose the one that is best for further in-vivo studies. For intra-articular drug delivery, the particle size of the microsphere should be below 10μm so that the particles can be readily phagocytosed by the macrophages of the inflamed synovium. The % entrapment efficiency of the carrier should be high so that the amount of the formulation that has to be injected is less and there is no wastage of the drug. Moreover, the microspheres should not be toxic to the synovium. The drug should be released in a controlled manner so that the synovium is not exposed to a significant amount of the drug at a time and the drug is slowly released to give an anti-inflammatory effect. Thus keeping these characteristics in mind, the formulations were characterized in-vitro for the entrapment efficiency, particle size and drug release. The surface morphology of the particles was studied by scanning electron microscopy and the cross-linking mechanisms were studied by FTIR.

5.2 Materials

Methanol, Dichloromethane, Hydrochloric acid, Sodium hydroxide, disodium hydrogen phosphate and potassium dihydrogen phosphate were purchased from S.D.Fine Chem. Ltd. Boisar, Thane. Glutaraldehyde was purchased from E.Merck India limited. Formaldehyde was purchased from S.D.fine chem.limited. Collagenase from clostridium histolyticum was purchased from Sigma Chemical Co. St., Louis, M.O.

5.3 Equipments

Ultracentrifuge, magnetic stirrer, Olympus microscope, Malvern particle size analyzer, FTIR, Scanning electron microscope, UV-Vis spectrophotometer.

5.4 % Entrapment efficiency

5.4.1 Gelatin microspheres

To weighed amount of gelatin microspheres, 2ml of 2N sodium hydroxide was added and allowed to stand for 24 hours to dissolve the microspheres. The solution was then extracted with 10ml x 2 dichloromethane and the organic extract evaporated to dryness. The residue was dissolved in methanol and the absorbance of the resulting solution was measured at the λmax of the drug. For celecoxib, the absorbance was measured at 250 nm, for rofecoxib 275 nm and for valdecoxib 237 nm using a Shimadzu UV-Vis spectrophotometer. The solutions containing rofecoxib were stored in amber colored volumetric flasks.

5.4.2 Chitosan microspheres

To weighed amount of chitosan microspheres, 2 ml of 0.1N hydrochloric acid was added and allowed to stand for 24 hours to degrade the microsphere matrix. The dispersion was then extracted with dichloromethane and the organic extract was evaporated to dryness. The residue was dissolved in methanol and the absorbance of the resulting solution was measured at the respective λmax of the drug.

5.4.3 Albumin microspheres

To weighed amount of albumin microspheres, 2 ml of 0.1N hydrochloric acid containing 2% w/v pepsin was added and allowed to stand for 24 hours. The dispersion was extracted with dichloromethane and the organic extract was evaporated to dryness. The residue was dissolved in methanol and the absorbance of the resulting solution was measured at the respective λmax of the drug.

5.4.4 Solid lipid nanoparticles

To weighed amount of solid lipid nanoparticles, 20 ml dichloromethane was added to extract celecoxib. The dispersion was filtered and the extract was evaporated to dryness. To the residue, 0.1N sodium hydroxide and filtered. After suitable dilutions, the absorbance of the resulting solution was measured at 250 nm using 0.1N sodium hydroxide as blank.

The above estimations gave the drug present in the microspheres.

The entrapment efficiency was then found out by the formula:

%Entrapment efficiency= (Drug present in the microspheres/Drug added) x 100.

Each estimation was done in triplicate.

5.5 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 or the nanoparticles dispersion were added to the sample dispersion unit containing the stirrer and stirred to reduce the interparticle aggregation and laser obscuration range was maintained at 15–20%. The average volume-mean particle size was measured after performing the experiment in triplicate.

5.6 Determination of residual formaldehyde or glutaraldehyde

Accurately weighed amount of microspheres were taken in a beaker and 10 ml distilled water was added. The mixture was shaken well and filtered using a whatman filter paper. The filtrate was analyzed for the residual formaldehyde or glutaraldehyde as per the method given in chapter 3.

5.7 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 for celecoxib and valdecoxib loaded

formulations. For rofecoxib loaded microspheres, phosphate buffer with 2.5% tween-80 was used as a 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 rpm in a thermostated bath at 37^0C. Samples (2 mL) were withdrawn at appropriate time intervals and centrifuged at 5000 rpm. Supernatants were diluted suitably and absorbance of the resulting solution was measured at the respective λmas of the drug in the dissolution medium. The residue was re-dispersed in 2mL of the fresh dissolution medium and replaced back into the vial. The release of the optimized batches was also studied in presence of collagenase. Collagenase was added to the release medium at a concentration of 50μg/ml. Calcium chloride was added as an activator of collagenase at a concentration of 50 μg/ml.

In-vitro release study of celecoxib from the solid lipid nanoparticles was done by dialysis bag diffusion method. The aqueous nanoparticles dispersion was placed in a cellulose dialysis bag (cutoff 12000, Himedia, India) and was sealed at both the ends. The dialysis bag was then immersed in the receptor compartment containing 100 ml of phosphate buffer pH-7.4 containing 2% tween-80 which is magnetically stirred at 50 rpm in a thermostated bath at 37°C. Samples were withdrawn at specific time intervals and were replaced by the fresh dissolution medium. The samples were suitably diluted and the absorbance of the resulting solutions was measured at 261 nm using dissolution medium as blank

5.8 Scanning electron microscopy studies

Scanning electron Microscopy of the microspheres was carried out to examine the surface morphology. The Microspheres were mounted on metal stubs and then coated with a 150 Å layer of gold. Photographs were taken using Jeol Scanning Electron Microscope (Jeol. JSM-5610LV SEM, Japan).

5.9 FTIR studies

FTIR spectral measurements were performed using a Shimadzu 8300 FTIR spectrometer. Microspheres were ground with KBr and FTIR spectra were taken in the range 4500-500cm^{-1}.

5.10 RESULTS AND DISCUSSION

5.10.1 Gelatin microspheres prepared by emulsification solvent-extraction method

No microspheres were formed in the absence of aluminium tristearate used as an anti-tacking agent. Instead, a hard unbreakable lump was obtained. In presence of aluminium tristearate, discrete microspheres were obtained in the form of a white fine powder. Thus, the role of aluminium tri-stearate was to avoid the agglomeration of the microspheres. Aluminium tri-stearate acts as a barrier to interparticle aggregation of the microdrops of the w/o emulsion. The results of the preliminary experiments revealed that at least 2.5% w/v of aluminium tristearate was necessary to obtain discrete microspheres. At lower concentrations, microspheres had a tendency to agglomerate. Even the absence of span-85 in the external phase led to agglomeration of the gelatin present in the internal phase of the w/o emulsion. Thus, both aluminium tristearate and span-85 are needed to obtain microspheres. Various solvents used to dehydrate the microspheres included isopropyl alcohol, acetone, mixture of alcohol and water etc. Discrete microspheres were obtained only when isopropyl alcohol or acetone were used as dehydrating agents. Mixtures of alcohol and water did not give discrete microspheres. As shown in table 5.1 very low entrapment efficiencies were obtained for celecoxib loaded gelatin microspheres prepared using emulsification solvent extraction method. The reason behind the low entrapment is the fact that celecoxib is highly soluble in isopropyl alcohol and thus when isopropyl alcohol or acetone is added, celecoxib which is associated with gelatin microdrops and present in

the internal phase of the emulsion gets solubilized. Thus, in addition to extracting water from the internal phase of the w/o emulsion, isopropyl alcohol or acetone also extracts celecoxib associated with the internal phase and hence very low entrapment efficiencies are obtained. As shown in table 5.1, increase in the gelatin concentration does not play a major role in preventing the dissolution of celecoxib in isopropyl alcohol and hence there is no significant difference in the entrapment efficiencies of the microspheres prepared using 15% or 25% gelatin.

Table 5.2 and table 5.3 shows the entrapment efficiencies of the rofecoxib and valdecoxib loaded gelatin microspheres by emulsification solvent extraction method. The solubility of rofecoxib in the isopropyl alcohol is less than that of celecoxib and thus significantly higher entrapment efficiency was obtained for rofecoxib. Thus, as shown in table 5.4, rofecoxib has higher entrapment in the gelatin microspheres compared to celecoxib. The solubility of valdecoxib in isopropyl alcohol is also less compared to the solubility of celecoxib and thus significantly higher entrapment efficiency was obtained for valdecoxib loaded gelatin microspheres. The effect of varying gelatin concentration and span-85 concentration on the entrapment efficiency and particle size was studied for rofecoxib and valdecoxib loaded gelatin microspheres. It was observed that gelatin concentration and span-85 concentration had no significant influence on the entrapment efficiency, while both gelatin concentration and span-85 had a significant influence on the particle size of the microspheres. Thus, it can be concluded that the main factor governing the entrapment efficiency is the solubility of the drug in the solvent which is used to dehydrate the microspheres. The drug which is having a low solubility in the extracting solvent will have higher entrapment efficiency, while the drug having a higher solubility has low entrapment efficiency.

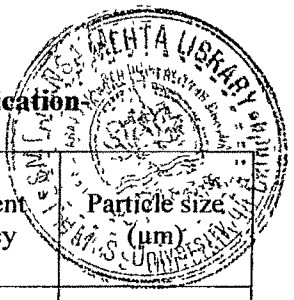


Table 5.1: Preparation of celecoxib loaded gelatin microspheres by emulsification-solvent extraction method

Batch code	Gelatin concentration (%w/w)	Span-85 concentration (%w/w)	Aluminium tri-stearate concentration (%w/v)	Observation	% Entrapment efficiency	Particle size (µm)
C-GM-1	15	0	0	No microspheres were formed	-	-
C-GM-2	15	2	0		-	-
C-GM-3	15	0	1.0		-	-
C-GM-4	15	2	1.0	No microspheres were formed	-	-
C-GM-5	15	2	2.5	Discrete microspheres were formed	15.63±1.77	22.62±2 26
C-GM-6	25	2	2.5	-do-	14 88±2.37	29.31±2.01
C-GM-7	25	5	2.5	-do-	16 27±2.22	25.85±3.11

Table 5.2: Preparation of rofecoxib loaded gelatin microspheres by emulsification solvent extraction method

Batch code	Gelatin concentration (%w/w)	Span-85 concentration (%w/w)	% Entrapment efficiency	Particle size (µm)
R-GM-1	15	1	35.61±3.03	25.53±2.03
R-GM-2	15	2.5	31.34±2.62	20.71±2.32
R-GM-3	15	5	30.73±1.85	18.52±1.65
R-GM-4	20	1	38.66±1.52	28.72±1.96
R-GM-5	20	2.5	40.05±0.47	26.44±1.18
R-GM-6	20	5	38.50±2.91	25.37±1.72
R-GM-7	25	1	39.41±1.91	30.73±3.24
R-GM-8	25	2.5	36.53±1.31	28.64±1.86
R-GM-9	25	5	39.62±2.44	29.30±1.70

Table 5.3: Preparation of valdecoxib loaded gelatin microspheres by emulsification solvent extraction method

Batch code	Gelatin concentration (%w/w)	Span-85 concentration (% w/w)	% Entrapment efficiency	Particle size (μm)
V-GM-1	15	1	40.65±1.75	25.38±0.75
V-GM-2	15	2.5	37.04±2.67	22.56±1.69
V-GM-3	15	5	38.51±2.47	20.44±3.34
V-GM-4	20	1	40.63±2.99	28.51±2.76
V-GM-5	20	2.5	39.84±2.33	27.37±1.17
V-GM-6	20	5	42.71±1.71	26.04±3.75
V-GM-7	25	1	44.33±2.19	32.04±2.16
V-GM-8	25	2.5	45.69±1.84	28.40±2.46
V-GM-9	25	5	42.83±2.58	27.66±2.50

5.10.2 Gelatin microspheres by emulsification chemical cross-linking method

The gelatin microspheres produced by this method are free flowing and shows no tendency to aggregate. In absence of polyethylene glycol, no discrete microspheres were formed. A thin jelly like material was obtained, which on drying was converted into hard unbreakable lumps. This indicated that there is an agglomeration of the gelatin microdrops present in the internal phase of the emulsion. This may be because, after the addition of the crosslinking agent, there is an inter-particle as well as intra-particle crosslinking of the gelatin droplets leading to agglomeration of the microparticles. The inter-particle cross-linking can be avoided by the use of de-aggregating agents, which provide a barrier to the coalescence of the microparticles. Various de-aggregating agents like magnesium stearate (Bogataj et al, 2000) and aluminium tri-stearate (Saparia et al, 2002) have been used in the preparation of microspheres. The disadvantage of using these agents is that they cannot be removed from the final formulation, they are not parenterally acceptable and they are known to

alter the dissolution properties of the drug. Hence, a parenterally acceptable material which acts as a de-aggregating agent and which can be removed from the final product is desirable to be used for the preparation of microspheres. Polyethylene glycol is used as an anti-tacking agent in the preparation of tablets. It is a non-ionic surfactant parenterally acceptable. It is also used as a protein micronization adjuvant in the preparation of gelatin (Morita et al, 2001) and albumin microspheres (Morita et al, 2000). Thus it was hypothesized that PEG-400 may act as a de-aggregating agent in the preparation of the microspheres. The choice of this particular grade of polyethylene glycol (PEG-400) is because of the miscibility of PEG-400 in the external phase as well as internal phase of the emulsion. Moreover, being water miscible, it can be easily removed from the final product by water washing. Microspheres were obtained as a fine powder when PEG-400 was added either in the external phase or in the internal phase of the emulsion. The formulations in which PEG-400 was added in the external phase of the emulsion had entrapment efficiency of only about 30-35%. It was confirmed that the entrapment efficiencies of the batches prepared by adding PEG-400 in the external phase of the emulsion were significantly lower ($p<0.05$) than the batches in which PEG-400 was added in the internal phase of the emulsion. Moreover, a significant decrease ($p<0.05$) in the entrapment efficiency with an increase in the PEG concentration in the external phase was also observed. This is because of the solubility of celecoxib in PEG-400. Though the concentration of PEG-400 employed was only 1-2%, it has a synergistic effect with SPAN-85 in solubilising celecoxib in the external phase of the emulsion. Thus very less entrapment efficiency was obtained. This was also confirmed by the presence of solubilized celecoxib in the external phase of the emulsion. Thus, further studies were carried out by adding PEG-400 in the internal phase of the emulsion. So,

PEG-400 was added in the internal phase of the emulsion as a de-aggregating agent. Addition of PEG-400 in the internal phase of the emulsion gave microspheres in the form of fine free-flowing powder. Since the solubility of celecoxib in the external phase comprising of liquid paraffin and span-85, is very less, high entrapment efficiencies were obtained. The effect of various factors on the entrapment efficiency, particle size and drug release characteristics of the microspheres was studied by varying the factor in consideration keeping other variables constant.

5.10.2a Entrapment efficiency and particle size

For determining the entrapment efficiency, it was necessary to degrade the microspheres matrix, so that the total drug associated with the microspheres could be determined. Determination of the entrapped drug was also carried out done by incubating the microspheres in methanol for 24 hours and then determining the drug present in the microspheres. But it was observed that incubation with methanol did not completely extract the drug from the microspheres. So, the microspheres were degraded by addition of 2N sodium hydroxide. Direct absorbance of this solution using 2N sodium hydroxide as blank could not be taken since there was interference from the excipients. So, the microspheres were dissolved in 2N sodium hydroxide and then the drug was extracted in dichloromethane. Since the calibration curves of the drugs were prepared in methanol, the dichloromethane extract was evaporated to dryness, the residue was dissolved in methanol and the absorbance of the resulting solution was taken to determine the drug present in the microspheres. The entrapment efficiency was then calculated by the formula given in section 5.4.

The effect of the various factors on the entrapment efficiency and the particle size was studied as follows:

5.10.2a.1 Effect of tween-80 concentration

Tween-80 was added in the gelatin solution to improve the wetting of the drugs, celecoxib, rofecoxib and valdecoxib. In absence of tween-80, it was not possible to prepare a uniform dispersion of the drugs in gelatin solution. It was observed that at least 2% tween-80 was required to obtain a uniform dispersion. The effect of concentration of tween-80 on the microspheres characteristics is shown in table 5.4 and it can be seen that there is no significant effect of tween-80 concentration on the particle size or entrapment efficiency of the microspheres.

Table 5.4: Effect of tween-80 concentration on the entrapment efficiency and particle size of celecoxib loaded gelatin microspheres

Tween-80 concentration (%w/w)	% Entrapment efficiency	Particle size (μm)
1%	86.02±1.45	20.35±1.84
2%	86.82±2.39	20.51±0.96
3%	87.30±1.71	22.36±1.31

Gelatin concentration=25%
Span-85 concentration=5.0%
Volume of glutaraldehyde=1.0 ml

5.10.2a.2 Effect of stirring speed

As shown in table 5.5, there is no significant difference in the entrapment efficiencies of the batches prepared using different stirring speeds indicating that stirring speed does not affect the entrapment efficiency. An increase in the stirring speed from 1500 rpm to 2500 rpm leads to a decrease in the particle size from 25.78μm to 20.51μm. The decrease in the particle size with an increase in the stirring speed is because higher stirring speed provides the required energy to the gelatin solution to be dispersed as fine droplets in the external oily phase thus giving smaller particle size with narrower size distribution. With further increase in

the stirring speed to 4000 rpm there is no significant decrease in the particle size. Thus 2500 rpm was selected as an optimum stirring speed.

Table 5.5: Effect of stirring speed on the entrapment efficiency and particle size of celecoxib loaded gelatin microspheres

Stirring speed (rpm)	% Entrapment efficiency	Particle size (μm)
1500	85.91±2.35	25.78±2.22
2500	86.82±2.39	20.51±0.96
4000	84.39±2.02	18.78±1.10

Gelatin concentration=25%
Span-85 concentration=5.0%
Volume of glutaraldehyde=1.0 ml

5.10.2a.3 Composition of external phase

The different compositions used were light liquid paraffin, heavy liquid paraffin and a mixture of light and heavy liquid paraffin (1:1). As shown in table 5.6, the oil phase did not affect the entrapment efficiency significantly. The geometric mean diameter of the microspheres prepared using light liquid paraffin was less than that of the mixture of light and heavy liquid paraffin and heavy liquid paraffin. But the microspheres prepared using light liquid paraffin and a mixture of light and heavy liquid paraffin was a mixture of particles of largely different sizes while microspheres prepared using heavy liquid paraffin was much uniform. Thus, heavy liquid paraffin was selected as an ideal external phase for the preparation of gelatin microspheres.

Table 5.6: Effect of composition of external phase on the entrapment efficiency and particle size of celecoxib loaded gelatin microspheres

Oil phase	% Entrapment efficiency	Particle size (μm)
Light liquid paraffin	87.82±2.17	16.84±2.13
Light liquid paraffin: Heavy liquid paraffin(1:1)	88.47±1.56	19.53±1.95
Heavy liquid paraffin	86.82±2.39	20.51±0.96

Gelatin concentration=25%
Span-85 concentration=5.0%
Volume of glutaraldehyde=1.0 ml

5.10.2a.4 Volume ratio of water: oil phase

The volume ratio of water: oil phase used were 1:20, 1:10 and 1:5. It was found that in 1:10 and 1:5 the microspheres tends to aggregate. Discrete microspheres were obtained when the water: oil phase was 1:20.

5.10.2a.5 Emulsification time

Three different emulsification times were chosen to study the effect of emulsification time on the characteristics of the microspheres. There was no significant effect of emulsification time on the entrapment efficiency of the microspheres. It was found that increase in the emulsification time from 5 minutes to 10 minutes led to a decrease in the particle size from 25.83μm to 20.51μm. Further increase in the emulsification time to 20 minutes led to agglomeration of the microspheres. Thus, 10 minutes was chosen as an optimum emulsification time.

5.10.2a.6 Effect of cross-linking agent

Two different cross-linking agents were used viz. formaldehyde and glutaraldehyde. The formaldehyde cross-linked microspheres were white in colour while the glutaraldehyde cross-linked microspheres were yellowish in colour. Both the microspheres were obtained as discrete fine powder. As shown in table 5.7, the volume of glutaraldehyde or formaldehyde does not have a significant influence on

the entrapment efficiency or the particle size of the microspheres. However, the particle size of the microspheres cross-linked using glutaraldehyde was found to be significantly lower ($p<0.05$) than that of the formaldehyde cross-linked microspheres. This may be because crosslinking with glutaraldehyde is reported to produce greater number and more stable cross-links than with formaldehyde (Oppenheim, 1987). The duration of cross-linking does not have a significant influence on the entrapment efficiency or the particle size.

Table 5.7: Effect of volume of glutaraldehyde (GA) or formaldehyde (FA) and duration of cross-linking on the entrapment efficiency and particle size

Batch code	Volume of Cross linking agent solution*(ml)	Duration of cross-linking (hours)	% Entrapment efficiency	Particle size (μm)
G-1	GA-0.5	1	84.78±1.80	22.62±1.72
G-2	GA-0.5	3	82.60±1.57	21.58±1.37
G-3	GA-1.0	1	84.80±2.58	22.65±1.09
G-4	GA-1.0	3	86.82±2.39	20.51±0.96
F-1	FA-0.5	1	85.30±1.74	28.94±1.40
F-2	FA-0.5	3	83.74±1.82	27.32±2.33
F-3	FA-1.0	1	86.77±1.91	27.60±1.15
F-4	FA-1.0	3	87.53±1.30	28.85±1.11

*GA= 25%w/w glutaraldehyde solution.
*FA=37%w/w formaldehyde solution.

5.10.2a.7 Effect of temperature of the external phase

Three different temperatures of the external phase were used to find out the optimum temperature for the preparation of the microspheres. When the temperature of the external phase was kept at room temperature, the particle size of the microspheres varied greatly may be because the gelatin dispersion was at 60°C and the external phase at room temperature, so a uniform dispersion was not

formed. The temperature of 60°C of the external phase gave more uniform particle size of the microspheres. At temperature of 80°C, the particles were uniform in size but the entrapment efficiency was reduced. This is due to the solubility of celecoxib in the external phase at higher temperature. Thus, 60°C was selected as an optimum temperature for the preparation of microspheres.

5.10.2a.8 Effect of gelatin concentration, span-85 concentration, volume of glutaraldehyde and Polyethylene glycol concentration

A 2^4 factorial design was used to investigate the combined effect of four different variables in the preparation of celecoxib loaded gelatin microspheres. The concentration of gelatin, concentration of span-85, volume of glutaraldehyde and concentration of Poly-ethylene glycol 400 were selected as causal factors while the particle size and the % entrapment efficiency were selected as the dependent variables. Two levels, low and high were selected for all the four factors. Potential variables such as the stirring speed, concentration of tween-80, volume ratio of oil: water phase, temperature of the external phase and emulsification time were kept constant in the experimental design. Based on the factorial design, sixteen batches of celecoxib loaded gelatin microspheres were prepared as shown in table 5.8. The main and the interaction effects of the variables on the entrapment efficiency and the particle size of the microspheres were studied. Mathematical modeling was carried out to obtain a polynomial equation. (full model, equation 1)(Anthony et al, 1996).

Table 5.8: Optimization of parameters for preparation of celecoxib loaded gelatin microspheres

Batch No.	Concentration of gelatin (%w/w)	Concentration of span-85 (%w/w)	Volume of glutaraldehyde (ml)	Concentration of PEG-400 (%w/w)	% Entrapment efficiency*	Particle size (μm)*
GMC-1	15	2	0.5	1	74.12	14.33
GMC-2	15	2	0.5	2	72.18	15.25
GMC-3	15	2	1.0	1	73.38	14.78
GMC-4	15	2	1.0	2	75.86	16.34
GMC-5	15	5	0.5	1	65.18	11.33
GMC-6	15	5	0.5	2	67.53	10.52
GMC-7	15	5	1.0	1	68.72	9.68
GMC-8	15	5	1.0	2	67.63	11.86
GMC-9	25	2	0.5	1	84.78	22.62
GMC-10	25	2	0.5	2	85.12	22.16
GMC-11	25	2	1.0	1	86.82	20.51
GMC-12	25	2	1.0	2	83.28	26.42
GMC-13	25	5	0.5	1	79.72	17.94
GMC-14	25	5	0.5	2	77.21	20.64
GMC-15	25	5	1.0	1	80.52	16.28
GMC-16	25	5	1.0	2	76.37	17.58

*The values are the mean of three results

Table 5.9: Coded values of the formulation parameters of celecoxib loaded gelatin microsphere by 2^4 factorial design

Coded values	Actual values			
	X1	X2	X3	X4
-1	15	2	0.5	1
+1	25	5	1.0	2

X1 = Concentration of gelatin %w/w
X2 = Concentration of span-85 %w/w
X3 = Volume of glutaraldehyde
X4 = Concentration of PEG-400

Table 5.10: 2^4 Factorial design layout of celecoxib loaded gelatin microspheres

Batch no.	X1	X2	X3	X4	X1X2	X1X3	X1X4	X2X3	X2X4	X3X4	X1X2X3X4	% Entrapment efficiency	Particle size (μm)
1	-1	-1	-1	-1	1	1	1	1	1	1	1	74.12	14.33
2	-1	-1	-1	1	1	1	-1	1	-1	-1	-1	72.18	15.25
3	-1	-1	1	-1	1	-1	1	-1	1	-1	-1	73.38	14.78
4	-1	-1	1	1	1	-1	-1	-1	-1	1	1	75.86	16.34
5	-1	1	-1	-1	-1	1	1	-1	-1	1	-1	65.18	11.33
6	-1	1	-1	1	-1	1	-1	-1	1	-1	1	67.53	10.52
7	-1	1	1	-1	-1	-1	1	1	-1	-1	1	68.72	9.68
8	-1	1	1	1	-1	-1	-1	1	1	1	-1	67.63	11.86
9	1	-1	-1	-1	-1	-1	-1	1	1	1	-1	84.78	22.62
10	1	-1	-1	1	-1	-1	1	1	-1	-1	1	85.12	22.16
11	1	-1	1	-1	-1	1	-1	-1	1	-1	1	86.82	20.51
12	1	-1	1	1	-1	1	1	-1	-1	1	-1	83.28	26.42
13	1	1	-1	-1	1	-1	-1	-1	-1	1	1	79.72	17.94
14	1	1	-1	1	1	-1	1	-1	1	-1	-1	77.21	20.64
15	1	1	1	-1	1	1	-1	1	-1	-1	-1	80.52	16.28
16	1	1	1	1	1	1	1	1	1	1	1	76.37	17.58

Table 5.11: Model coefficients estimated by multiple linear regression for celecoxib loaded gelatin microspheres by 2^4 factorial design

FACTOR	COEFFICIENTS	COMPUTED T-VALUE	P-VALUE*
Intercept	76.151	253.795	1.446E-09
X1	5.576	18.584	4.9342E-05
X2	-3.295	-10.969	0.0004
X3	0.421	1.403	0.233
X4	-0.503	-1.678	0.168
X1X2	0.018	0.062	0.953
X1X3	-0.401	-1.337	0.252
X1X4	-0.728	-2.428	0.072
X2X3	0.028	0.095	0.928
XX2X4	-0.171	-0.570	0.598
X3X4	-0.283	-0.945	0.397
X1X2X3X4	0.631	2.103	0.103

*Significant at $p<0.05$

Table 5.12: Analysis of variance (ANOVA) of full and reduced models of celecoxib loaded gelatin microspheres by 2^4 factorial design

		DF	SS	MS	F	R	R^2	Adj R^2
Regression	FM	11	696.95	63.35	43.98	0.995	0.991	0.969
	RM	2	670.83	335.41	136.74	0.977	0.954	0.947
Error	FM	4	5.76(E1)	1.44	43.98			
	RM	13	31.88(E2)	2.45	136.74			

SSE2-SSE1 – 31.88- 5.76 = 26.12
No.of parameters omitted = 9
MS of error (full model) = 1.44
F calculated = (26.12/9)/1.44 = 2.01

Table 5.13: Model coefficients estimated by multiple linear regression for celecoxib loaded gelatin microspheres by 2^4 factorial design(particle size)

Factor	Coefficients	Computed t-value	p- value*
Intercept	16.765	71.379	2.31E-07
X1	3.753	15.982	8.96E-05
X2	-2.286	-9.734	0.000624
X3	-0.083	-0.356	0.739
X4	0.831	3.539	0.024
X1X2	-0.122	-0.521	0.629
X1X3	-0.2375	-1.011	0.369
X1X4	0.350	1.490	0.210
X2X3	-0.545	-2.320	0.08
XX2X4	-0.160	-0.681	0.533
X3X4	0.537	2.288	0.083
X1X2X3X4	-0.632	-2.692	0.054

***Significant at p<0.05**

Table 5.14: Analysis of variance (ANOVA) of full and reduced models of celecoxib loaded gelatin microspheres by 2^4 factorial design (Particle size)

		DF	SS	MS	F	R	R^2	Adj R^2
Regression	FM	11	339.53	30.86	34.97	0.994	0.989	0.961
	RM	3	320.13	106.71	55.84	0.966	0.933	0.916
Error	FM	4	3.53	0.88	34.97			
	RM	12	22.93	1.91	55.84			

SSE2-SSE1=22.93-3.53=19.40
No. of parameters omitted=8
MS of error (full model)=0.88
F calculated= (19.40/8)/0.88=2.75

The % entrapment efficiency obtained at various levels of four independent variables (X1, X2, X3, and X4) was subjected to multiple linear regression to yield a second order polynomial equation (full model. Equation 1)

Y= 76.75+ 5.57 X1 -3.29 X2 +0.421 X3-0.503 X4+0.018 X1X2-0.401X1X3-0.728 X1X4+ 0.028 X2X3 -0.171 X2X4 – 0.283 X3X4 +0.631 X1X2X3X4.....(Equation 1)

The main effects of X1, X2, X3, X4 represent the average result of changing one variable at a time from its low to high value. The interactions(X1X2, X1X3, X1X4, X2X3, X2X4, X3X4, and X1X2X3X4) show how the entrapment efficiency changes when two or more variables are simultaneously changed. The entrapment efficiency for the sixteen batches shows a wide variation from 65.18% to 86.82%. Small values of coefficients in terms of X3, X4, X1X2, X1X3, X1X4, X2X3, X2X4 and X1X2X3X4 are regarded as least contributing in the preparation of celecoxib loaded gelatin microspheres. Hence these non-significant terms are neglected from the full model and a reduced polynomial equation (equation 2) is obtained following multiple regression of entrapment efficiency and very significant ($p<0.05$) terms of equation 1.

Y= 76.15 + 5.57X1 – 3.29X2..... (Equation 2)

The significance of each coefficient of the equation 1 was determined by student't' test and p-value which showed that the quadratic main effects of the concentration of gelatin (p value= 0.000049) and concentration of span-85(p=0.00039) are found to be extremely significant. The interaction between the different variables is not significant as evidenced from their p values.

ANOVA between the full and the reduced model was performed. F-statistic of the results of the Full and reduced model confirmed omission of non-significant terms of equation 1. When the coefficients of the four independent variables in equation 1 were compared, the value of the variable X1(b1=5.57) and X2(b2=3.29) was found to be

maximum and hence both the variables, concentration of gelatin and concentration of span-85 were considered to be major contributing variables for entrapment efficiency of celecoxib microspheres. The goodness of fit of the model was checked by the determination of coefficient (R^2). In this case, the values of the determination coefficients (R^2 =0.991 for the full model and 0.954 for the reduced model) indicated that over 90% of the total variations are explained by the model. The values of adjusted determination coefficients (adj R^2 = 0.969 for full model and 0.947 for reduced model) are also very high which indicates a high significance of the model. All the above considerations indicate and excellent adequacy of the regression model (Adinarayana et al, 2002, Box et al, 1978, Cochran and Cox, 1992).

The particle size obtained for the various batches was subjected to multiple linear regression to yield a polynomial equation (equation 3, full model)

Y=16.76+3.75X1-2.28X2-0.008X3+0.83X4-0.12X1X2-0.23X1X3+0.35X1X4-0.54X2X3-0.16X2X4+0.53X3X4-0.63X1X2X3X4.....(Equation 3)

The geometric mean diameter of the sixteen batches shows a variation from 9.68μm to 26.48μm. Small values of coefficients in terms of X3, X1X2, X1X3, X1X4, X2X3, X2X4 and X1X2X3X4 are regarded as least contributing in the preparation of celecoxib loaded gelatin microspheres. Hence these non-significant terms are neglected from the full model and a reduced polynomial equation (equation 4) was obtained following multiple regression of entrapment efficiency and very significant ($p<0.05$) terms of equation 3.

Y= 16.76 + 3.75X1 – 2.28X2+0.83X4.....(Equation 4)

The significance of each coefficient of the equation 1 was determined by student't' test and p-value which showed that the quadratic main effects of the concentration of gelatin (p value= 0.00009) and concentration of span-85(p=0.0006) are found to be

extremely significant. The interaction between the different variables is not significant as evidenced from their p values.

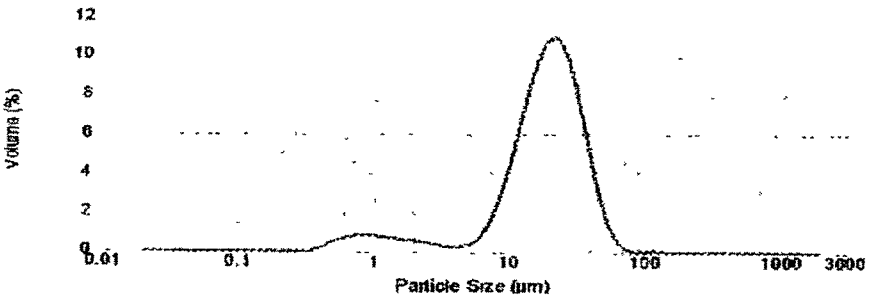
ANOVA between the full and the reduced model was performed. F-statistic of the results of the Full and reduced model confirmed omission of non-significant terms of equation 2. When the coefficients of the four independent variables in equation 1 were compared, the value of the variable X1(b1=3.75), X2(b2=2.28), X4(b4=0.83) was found to be maximum and hence the three variables, concentration of gelatin, concentration of span-85 and concentration of Polyethylene glycol were considered to be major contributing variables for particle size of gelatin microspheres. The effect of concentration of polyethylene glycol was not as significant as that of the concentration of gelatin and span-85 as evidenced by the p values and the coefficient value. The goodness of fit of the model was checked by the determination of coefficient (R^2). In this case, the values of the determination coefficients R2=0.989 for the full model and 0.933 for the reduced model indicated that over 90% of the total variations are explained by the model. The vales of adjusted determination coefficients (adj R^2 = 0.961 for full model and 0.916 for reduced model) are also very high which indicates a high significance of the model. All the above considerations indicate and excellent adequacy of the regression model (Adinarayana et al, 2002, Box et al, 1978, Cochran and Cox, 1992).

Thus from the results of the factorial design, it can be inferred that out of the four factors studied, concentration of gelatin and span-85 are the factors which affect the particle size and entrapment efficiency of the microspheres. The PEG-400 concentration in the internal phase and the volume of glutaraldehyde had no significant influence on the entrapment efficiency and particle size. An increase in the gelatin concentration from 15% w/w to 25% w/w led to an increase in the entrapment

efficiency from 75% to 86%. The increase in the entrapment efficiency with an increase in the gelatin concentration was due to the formation of more viscous solutions with an increase in gelatin concentration. Similar results were obtained by previous workers (Sankar and Mishra, 2003). With an increase in the viscosity, more amount of drug is associated with the gelatin and less is present as free drug. The decrease in entrapment efficiency with a decrease in the gelatin concentration is because at lower concentration of gelatin, more amount of drug is present as free drug. The decrease in the entrapment efficiency with an increase in the SPAN-85 concentration is due to increase in the solubility of celecoxib in the external phase of the emulsion. This effect is more pronounced at lower concentration of gelatin, which indicates that the solution with less viscosity can less efficiently prevent the dissolution of the drug in the external phase.

There was an increase in the particle size of the microspheres with an increase in the gelatin concentration whereas a decrease in the particle size was observed with an increase in the span-85 concentration. An increase in the particle size of the microsphere with an increase in the gelatin concentration is due to the formation of bigger droplets of the internal phase in the emulsification step for the preparation of microspheres, because of increase in the viscosity. There is a decrease in the interfacial tension between the aqueous internal phase and the oily external phase with an increase in the SPAN-85 concentration. This leads to a decrease in the particle size with an increase in the SPAN-85 concentration. The particle size distribution of the celecoxib loaded gelatin microspheres (figure 5.1) shows that the particles have fairly uniform size.

Figure 5.1: Particle size distribution of celecoxib loaded gelatin microspheres



From the data obtained for the celecoxib loaded gelatin microspheres, it was observed that the main factors affecting the entrapment efficiency and the particle size of the microspheres are the concentration of gelatin and concentration of span-85. So various batches of the rofecoxib and valdecoxib loaded gelatin microspheres were prepared by varying these two factors. The effect of these two factors on the characteristics of the rofecoxib and valdecoxib loaded gelatin microspheres was studied by varying these factors and keeping other factors constant. The composition of the various batches of rofecoxib and valdecoxib loaded gelatin microspheres and their entrapment efficiencies and particle size is shown in tables 5.15 and 5.16 respectively.

Table 5.15: Effect of concentration of gelatin and concentration of span-85 on the entrapment efficiency and particle size of rofecoxib loaded gelatin microspheres

Batch code	Concentration of gelatin (%w/w)	Concentration of span-85 (%w/w)	Volume of glutaraldehyde (ml)	% Entrapment efficiency	Particle size (μm)
R-GM1	15%	2	0.5	65.52±1.43	25.61±1.65
R-GM2	15%	2	1.0	66.77±3.35	24.83±1.97
R-GM3	15%	5	0.5	63.84±2.27	20.41±1.89
R-GM4	15%	5	1.0	65.03±1.54	19.37±1.19
R-GM5	25%	2	0.5	81.26±0.92	29.52±1.60
R-GM6	25%	2	1.0	80.37±2.09	28.61±1.91
R-GM7	25%	5	0.5	79.52±1.90	25.74±0.91
R-GM8	25%	5	1.0	79.4±2.02	24.38±1.39

Table 5.16: Effect of concentration of gelatin, concentration of span-85 on the entrapment efficiency and particle size of valdecoxib loaded gelatin microspheres

Batch code	Concentration of Gelatin (%w/w)	Concentration of span-85 (%w/w)	Volume of glutaraldehyde (ml)	% Entrapment efficiency	Particle size (μm)
V-GM1	15%	2	0.5	78.51±1.82	17.25±2.93
V-GM2	15%	2	1.0	80.73±2.11	19.04±1.85
V-GM3	15%	5	0.5	70.42±2.73	14.47±1.93
V-GM4	15%	5	1.0	71.47±2.36	13.58±2.10
V-GM5	25%	2	0.5	93.16±2.16	21.73±2.48
V-GM6	25%	2	1.0	95.4±1.66	22.47±2.14
V-GM7	25%	5	0.5	92.11±1.80	19.84±0.96
V-GM8	25%	5	1.0	90.63±1.93	20.50±1.03

As shown in table 5.15, high entrapment efficiencies were obtained for rofecoxib loaded gelatin microspheres. Since rofecoxib is a light sensitive drug, rofecoxib loaded microspheres were protected from direct light. As shown in table 5.15, there is no significant effect ($p>0.05$) of span-85 concentration on the entrapment efficiency of the microspheres. The reason behind this finding is the insolubility of rofecoxib in the external phase of the emulsion. Thus, increase in the span-85 concentration did not decrease the entrapment efficiency by bringing about solubilization of rofecoxib in the external phase of the emulsion. The span-85 concentration had a significant influence on the particle size of the microspheres. With an increase in the span-85 concentration from 2%w/w to 5%w/w, there was a significant decrease in the particle size.

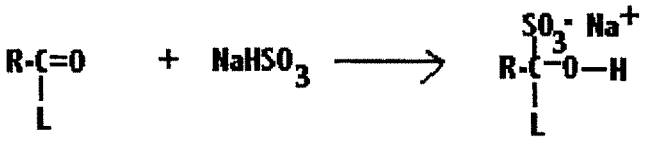
The concentration of gelatin had a significant influence on the entrapment efficiency of the microspheres. At low concentration of gelatin, more amount of rofecoxib was in the free form. As the concentration of gelatin was increased from 15% to 25%,

more amount of rofecoxib was associated with the gelatin and hence higher entrapment efficiency was obtained. All the batches prepared using 25% gelatin shows high entrapment efficiency of around 80%.

The entrapment efficiencies and particle size of the different batches of valdecoxib loaded gelatin microspheres is shown in table 5.16. The valdecoxib loaded gelatin microspheres shows high entrapment efficiency ranging from 70.42% to 95.40%. As shown in table 5.16, the concentration of gelatin and concentration of span-85 have a significant influence on the entrapment efficiency as well as particle size of the microspheres. The volume of glutaraldehyde has no significant influence on the particle size or the entrapment efficiency of the microspheres. There is a decrease in the entrapment efficiency with an increase in the span-85 concentration which is because of the solubility of valdecoxib in the external phase of the emulsion. There is an increase in the entrapment efficiency with an increase in the gelatin concentration.

5.10.2b Residual glutaraldehyde and formaldehyde content

The residual glutaraldehyde and formaldehyde content in all the formulations was found to be less than 5 ppm. The reason behind this is that the microspheres are washed with sodium bisulphite solution which neutralizes the free aldehyde groups. As shown in the following reaction, 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.



Thus, the toxicity of the residual glutaraldehyde or formaldehyde is not of a major concern in this formulation.

5.10.2c Drug release

Since, celecoxib, rofecoxib and valdecoxib were not soluble in phosphate buffer pH 7.4, it was necessary to use a dissolution media in which the drugs are soluble and also sink conditions are maintained. Hydroalcoholic mixtures are used as dissolution media in the cases where the drug is poorly soluble in plain buffer. Our studies indicated that the drugs are not soluble in presence of upto 30% of alcohol. Also, the alcohol could possibly dehydrate the microspheres leading to the formation of fractures on the microspheres surface. Addition of surfactant to the dissolution media is a better approach to increase the solubility of the drug. Sodium lauryl sulphate is a commonly used surfactant in the dissolution medium. The release studies carried out in presence of SLS resulted in immediate release of the drug within 1 hour. After the release test, when the microspheres were observed under optical microscope, the microspheres had distorted structures which indicate that sodium lauryl sulphate is responsible for degrading the microspheres. Thus, further release tests were done by adding tween-80 in the dissolution medium. From the preliminary studies, we concluded that 2% w/w tween-80 is needed to maintain sink conditions in case of celecoxib and valdecoxib microspheres while 2.5% tween-80 is needed for rofecoxib microspheres. The release study of the microspheres was also conducted in presence of collagenase. It was necessary to add calcium chloride as an activator for collagenase. The previous workers also have added calcium chloride in the release medium to activate collagenase so that it degrades the microspheres (Mladenovska et al, 2002).

The effect of the different variables on the in-vitro release profiles of celecoxib loaded gelatin microspheres is shown in tables 5.17 to 5.20 and figures 5.2 to 5.6.

Table 5.17: Effect of volume of glutaraldehyde and duration of cross-linking on the release of celecoxib from gelatin microspheres

Time(h)	Cumulative % release			
	Batch G-1 (0.5ml-1 hour)	Batch G-2 (0.5ml-3 hours)	Batch G-3 (1.0 ml-1 hour)	Batch G-4 (1.0ml-3 hours)
1	50.94±1.81	46.72±1.80	44.76±2.53	38.63±2.58
2	56.7±1.61	52.47±1.91	50.51±2.19	43.7±2.80
3	62.56±2.17	57.91±1.27	54.02±1.61	45.61±2.76
4	69.4±1.64	63.83±2.73	59.33±2.28	50.17±2.13
8	72.49±2.90	68.38±2.88	65.83±2.50	60.59±1.54
24	85.71±1.96	80.96±2.30	73.94±2.20	68.62±2.86
48	94.33±1.82	92.7±2.66	81.88±2.38	73.08±2.44
72			92.49±1.73	85.52±1.54
96			97.53±1.57	93.6±2.25

Figure 5.2: Effect of volume of glutaraldehyde (25%w/w) and duration of cross-linking on the drug release

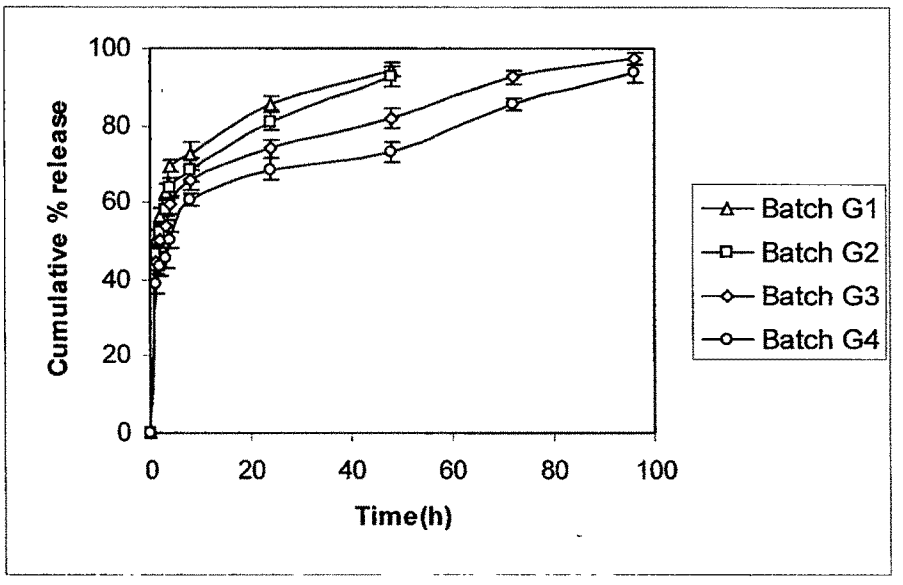


Figure 5.4: Comparative release profiles of formaldehyde (Batch F4) and Glutaraldehyde (Batch G-4) crosslinked gelatin microspheres

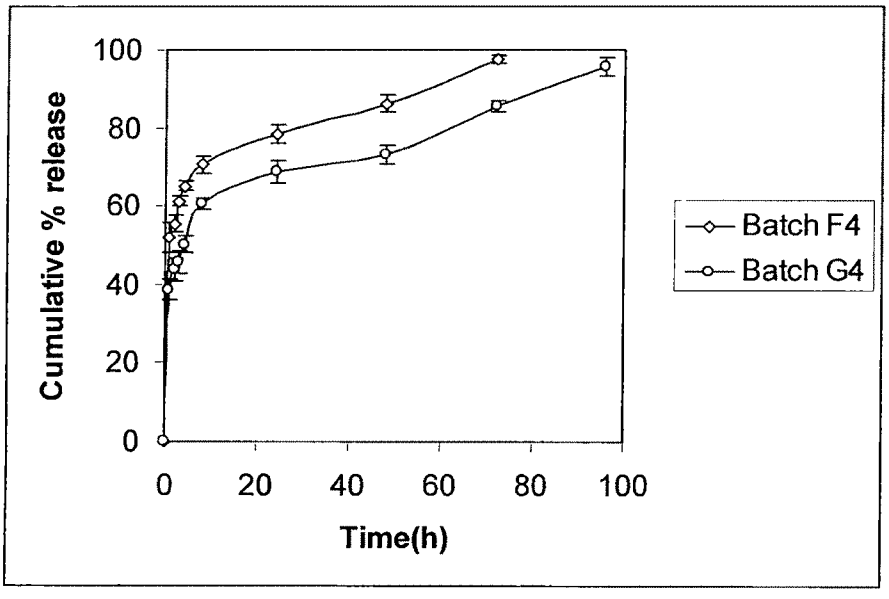


Table 5.19: Effect of gelatin concentration on release of celecoxib from gelatin microspheres

Time(h)	Cumulative % release	
	Batch GMC-3 (15% w/w)	Batch GMC-11 (25% w/w)
1	50.76±2.40	38.63±2.58
2	58.93±2.27	43.7±2.80
3	62.75±2.42	45.61±2.76
4	70.48±2.57	50.17±2.13
8	79.2±2.17	60.59±1.54
24	88.63±2.57	68.62±2.86
48	96.18±2.33	73.08±2.44
72		85.52±1.54
96		95.6±2.25

Figure 5.5: Effect of gelatin concentration on release of celecoxib from gelatin microspheres

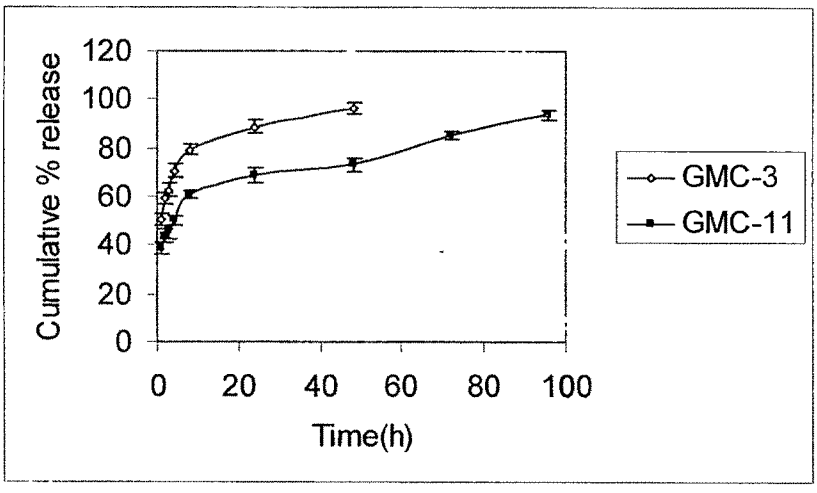


Table 5.20: Effect of presence of collagenase in the dissolution medium on the release of celecoxib from gelatin microspheres

Time(h)	Cumulative % release of celecoxib from gelatin microspheres(Batch G-4)	
	Without collagenase	With collagenase
1	38.63±2.58	50 74±1.85
2	43.7±2.80	56 63±1.27
3	45.61±2.76	61.36±2.65
4	50.17±2.13	68.95±1.39
8	60.59±1.54	75 38±1.82
24	68.62±2.86	83.19±2.45
48	73.08±2.44	92.24±1.32
72	85.52±1.54	98 4±1.37
96	95.6±2.25	

Figure 5.6: Effect of presence of collagenase in the dissolution medium on the release of celecoxib from gelatin microspheres

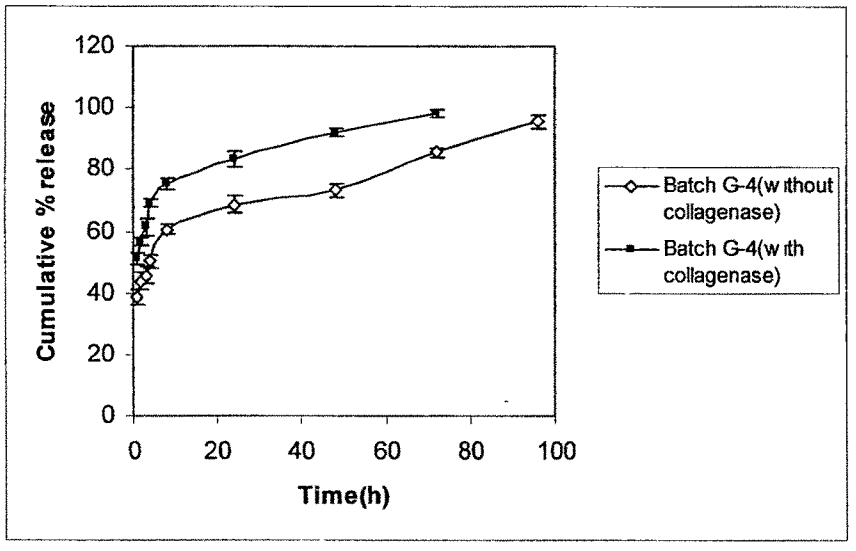


Table 5.21: In-vitro release profile of celecoxib loaded gelatin microspheres (fitted to Korsemeyer-peppas model (Rao and Shyale, 2004)

Log t	Log(Qt/Qα)									
	G1	G2	G3	G4	F1	F2	F3	F4	GMC-11	G4 (with Collagenase
0	-0.292	-0.330	-0.349	-0.413	-0.216	-0.254	-0.272	-0.285	-0.294	-0.295
0.301	-0.246	-0.280	-0.296	-0.359	-0.181	-0.232	-0.216	-0.255	-0.229	-0.247
0.477	-0 203	-0.237	-0.267	-0.340	-0 140	-0.167	-0.175	-0.212	-0.202	-0.212
0.602	-0.158	-0.194	-0.226	-0.299	-0.114	-0.140	-0.144	-0.186	-0 151	-0.161
0.903	-0.139	-0.165	-0.181	-0.217	-0.066	-0.085	-0.105	-0.152	-0.101	-0.123
1.380	-0.066	-0.091	-0.131	-0.163	-0.019	-0.042	-0.072	-0.105	-0.052	-0.080
1.681	-0.025	-0.032	-0.086	-0.136		-0.011	-0.019	-0.064	-0.016	-0.035
1.857			-0.033	-0.067				-0.0099		-0.007
1 982			-0.010	-0.028						

Table 5.22: Release kinetic parameters of celecoxib loaded gelatin microspheres

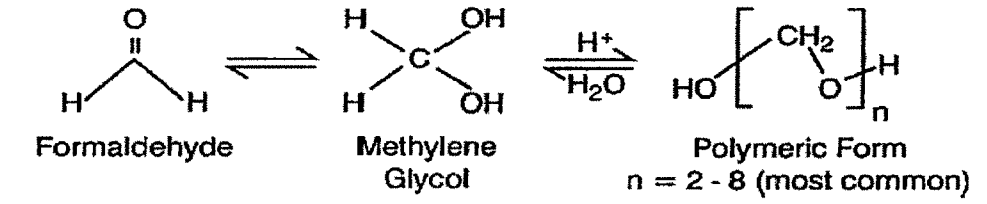
Batch code	Correlation coefficient (r^2)				n (peppas model)	K (peppas model)
	Zero order	Higuchi	First order	Peppas		
G1	0.823	0.930	0.975	0.979	0.157	0.522
G2	0.863	0.955	0.981	0.988	0.173	0.476
G3	0.897	0.970	0.968	0.989	0.162	0.456
G4	0.903	0.966	0.960	0.982	0.184	0.386
F1	0.793	0.915	0.970	0.975	0.148	0.610
F2	0.747	0.879	0.969	0.956	0.150	0.564
F3	0.787	0.898	0.960	0.963	0.142	0.559
F4	0.896	0.963	0.928	0.983	0.139	0.520
GMC-11	0.762	0.892	0.966	0.967	0.163	0.530
G-4(with collagenase	0.817	0.923	0.941	0.975	0.149	0.525

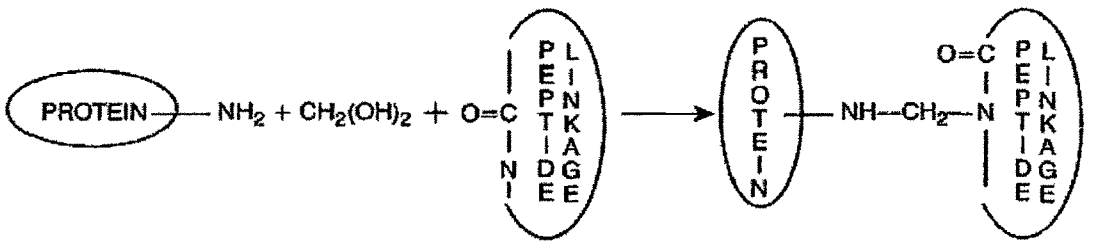
n=release exponent
K= release rate constant

The in-vitro drug release studies indicated that the main factors affecting the release of celecoxib from the microspheres were the volume of the cross-linking agent, duration of cross-linking and concentration of gelatin. The stirring speed, emulsifier concentration and PEG concentration had no significant influence on the drug release. As shown in figure 5.2 and figure 5.3, an increase in the volume of the cross-linking agent (glutaraldehyde/formaldehyde) led to a decrease in the rate of drug release. Microspheres prepared using 1.0 ml of the cross-linking agent releases the drug slowly compared to the microspheres in which 0.5 ml of the cross-linking agent was used. However, a burst effect was observed in all the formulations. This burst effect is attributed to the drug crystals present on the surface of the microspheres. Similar results were obtained by previous workers (Muvaffak et al, 2004). This has been confirmed by the scanning electron micrographs which show that the surface of the microsphere is somewhat rough with drug crystals present on the surface. In general, around 40% of the drug is released in the first hour, followed by slower release of the remaining drug over a period of 96 hours (Figure 5.2) in case of glutaraldehyde cross-linked microspheres. In case of formaldehyde crosslinked microspheres about 55% of the drug is released in the first hour followed by a controlled release for a period of 72 hours (figure 5.3). Figure 5.4 shows a comparative release profile of glutaraldehyde and formaldehyde cross-linked microspheres. There is a significant difference ($p<0.05$) in the drug release rates of the formaldehyde cross-linked microspheres and the glutaraldehyde cross-linked microspheres. The formaldehyde cross-linked microspheres showed significantly higher ($p<0.05$) release rates than the glutaraldehyde cross-linked microspheres. The reason behind this finding may be the greater number and more stable cross-links produced by glutaraldehyde than with

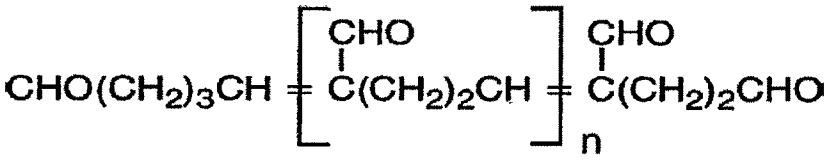
formaldehyde. The reaction mechanism of the formaldehyde as well as glutaraldehyde with gelatin is shown below:

Reaction mechanism of formaldehyde with gelatin

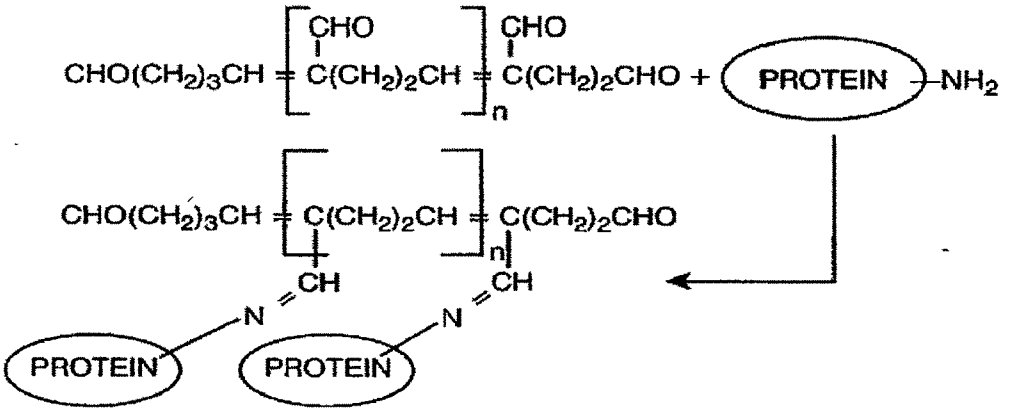




Reaction mechanism of glutaraldehyde with gelatin



Polymeric form of glutaraldehyde



The duration of cross-linking also had an effect on the drug release. But this effect was less pronounced than that of the volume of the cross-linking agent. An increase in the duration of cross-linking from 1 hour to 3 hours led to a decrease in the drug release as shown in Figures 5.2 & 5.3 respectively for glutaraldehyde and formaldehyde cross-linked microspheres.

There was a decrease in the drug release with an increase in the concentration of gelatin. As shown in figure 5.5, the microspheres prepared using 25% gelatin (Batch GMC-11) releases the drug slowly compared to the microspheres prepared using 15% gelatin (Batch GMC-3). The reason behind this finding may be the microspheres prepared using 25% gelatin when comes in contact with the dissolution medium, produces a more rigid microdrop and hence the drug is slowly released.

To examine the kinetics of drug release and mechanism, the release data were fitted to models representing zero order, first order, Higuchi's square root of time (Sankar and Mishra, 2003) and Korsemeyer and peppas model. The coefficient of correlation values (Calculated from the plots of Q vs t for zero order, Log(Qo-Q) vs t for first order and Q vs $t^{1/2}$ for Higuchi model, log(Q/Qα) vs. log t for peppas model where Q is the amount of drug released at time t, Qα is the amount of drug released at time α and Qo-Q is the amount of drug remaining after time t) were highest (except batch F2) in case of korsemeyer and peppas model. Thus it can be concluded that the celecoxib release from the microspheres is best explained by Korsemeyer and peppas model. The mechanism of the drug release was further investigated by the korsemeyer and peppas equation:

Log(Qt/Qα)= log k + n log t, where, Qt is the amount of drug released at time t and Qα is the amount of drug released at time α., K is the kinetic constant characterizing the polymeric system, and n is the release exponent. When $n \leq 0.5$, this indicates a

quasi fickian diffusion mechanism, when n>0.5, an anamolous non-fickian diffusion is observed, whereas n=1 indicates a zero order release (Sankar et al, 2003). The values of n and K were obtained from the plot of log (Qt/Qα) vs. log t. The values of n obtained for all the batches are less than 0.5, which indicates that the drug is released by quasi fickian diffusion.

In order to study the effect of collagenase on the drug release rates, the release studies of the optimized batch G-4 was conducted by adding collagenase in the release medium. It can be seen from table 5.20 and figure 5.6 that the release rate of celecoxib increases in the presence of collagenase. However, the presence of calcium is necessary for the activation of collagenase. In absence of calcium, no significant difference was observed between the release rates in presence or absence of collagenase. Calcium chloride was added in the release medium to activate collagenase. The presence of collagenase in the release medium leads to degradation of the microspheres and hence the release rates are increased. Similar results were obtained by previous workers (Tabata and Ikada, 1989).

The in-vitro release data of celecoxib loaded gelatin microspheres shows that the main factors affecting the drug release from the microspheres are the concentration of gelatin and volume of the cross-linking agent. The glutaraldehyde crosslinked microsphere seems to be more promising than the formaldehyde crosslinked microspheres because of their slower drug release rates. So for preparation of rofecoxib and valdecoxib loaded gelatin microspheres, glutaraldehyde was used as a crosslinking agent. The effect of glutaraldehyde volume, duration of cross-linking and concentration of gelatin on the drug release was studied by varying these factors and performing the drug release studies as indicted earlier. The effect of these variables on the release of rofecoxib from gelatin microspheres is shown in tables 5.23 and 5.24

and figures 5.7 and 5.8. The release kinetic parameters of the rofecoxib loaded gelatin microspheres is shown in table 5.26.

Table 5.23: Effect of volume of glutaraldehyde and duration of cross-linking on the drug release from rofecoxib loaded gelatin microspheres

Time(h)	Cumulative % release			
	R-GM7-1 hour (0.5ml)	R-GM7-3 hours (0.5 ml)	R-GM8-1 hour (1.0ml)	R-GM8- 3 hours (1.0 ml)
1	62.35±1.20	60.47±1.79	56.63±1.60	52.27±2.33
2	68.03±2.05	67.16±2.02	60.74±1.51	58.63±1.94
3	74.51±1.32	72.38±2.96	64.58±1.40	65. 2±2.30
4	79.62±2.28	78.61±2.14	67.21±1.13	69.38±1.04
8	84.5±1.94	85.81±1.04	72.19±1.29	73.84±2.38
24	95.37±1.84	97.55±2.22	80.43±2.06	79.61±2.71
48			85.29±1.55	83.42±0.56
72			93.46±1.84	89.36±1.77
96				95.51±1.53

Figure 5.7: Effect of volume of glutaraldehyde and duration of cross-linking on the drug release from rofecoxib loaded gelatin microspheres

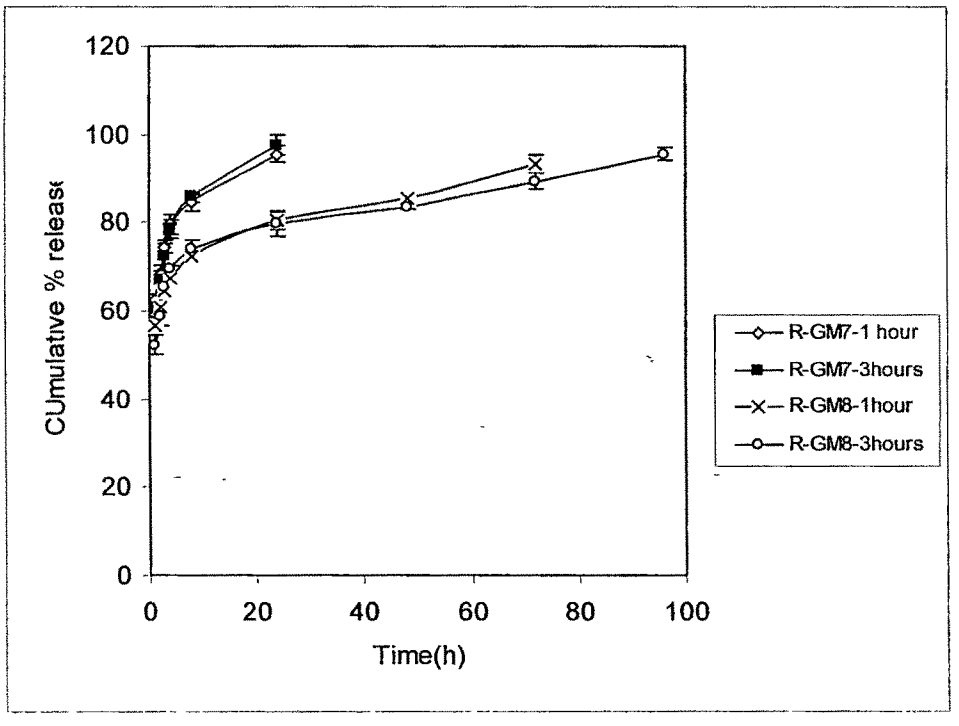


Table 5.24: Effect of gelatin concentration on the release of rofecoxib from gelatin microspheres

Time(h)	Cumulative % release	
	R-GM4 (15%w/w gelatin)	R-GM8 (25%w/w gelatin)
1	70.52±1.26	56.63±1.60
2	74.36±1.25	60.74±1.51
3	79.15±1.77	64.58±1.40
4	83.08±1.27	67.21±1.13
8	88.26±0.90	72.19±1.29
24	93.71±2.17	80.43±2.06
48	98.63±1.85	85.29±1.55
72		93.46±1.84

Figure 5.8: Effect of gelatin concentration on the release of rofecoxib from gelatin microspheres

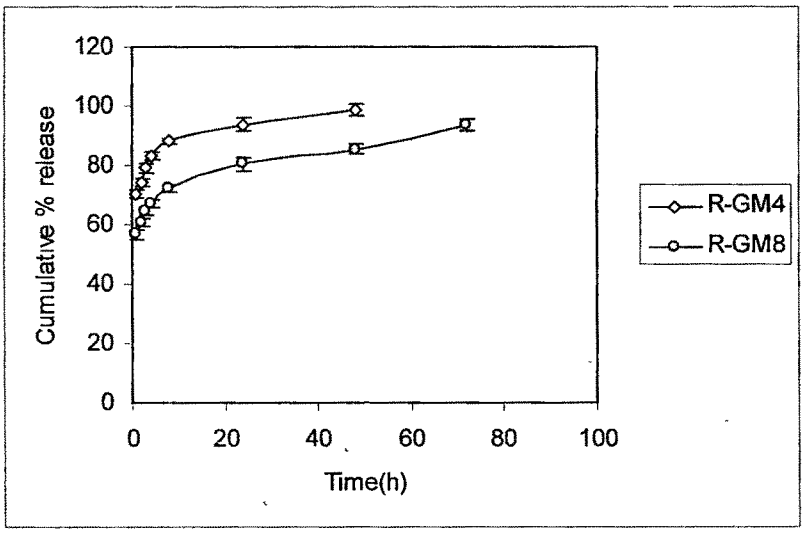


Table 5.25: In-vitro release profile of rofecoxib loaded gelatin microspheres (fitted to Korsemeyer-peppas model)

Log t	Log(Qt/Qα)				
	R-GM7-1 hour	R-GM7-3 hours	R-GM8-1 hour	R-GM8-3 hours	R-GM4
0.000	-0.205	-0.218	-0.247	-0.282	-0.152
0.301	-0.167	-0.173	-0.217	-0.232	-0.129
0.477	-0.128	-0.140	-0.190	-0.186	-0.102
0.602	-0.099	-0.105	-0.173	-0.159	-0.081
0.903	-0.073	-0.066	-0.142	-0.132	-0.054
1.380	-0.021	-0.011	-0.095	-0.099	-0.028
1.681			-0.069	-0.079	-0.006
1.857			-0.029	-0.049	
1.982				-0.020	

Table 5.26: Release kinetic parameters of rofecoxib loaded gelatin microspheres

Batch code	Correlation coefficient (r^2)				n (peppas model)	K (peppas model)
	Zero order	Higuchi	First order	Peppas		
R-GM7-1 hour	0.784	0.904	0.969	0.973	0 135	0.635
R-GM7-3 hours	0.805	0.922	0.991	0.983	0.154	0.613
R-GM8-1 hour	0 878	0.961	0 967	0.993	0.111	0.568
R-GM8-3 hours	0.810	0.904	0.944	0.957	0.116	0.555
RGM4	0.748	0.879	0.975	0.970	0.087	0.716

n= Release exponent
K=Release rate constant

As shown in table 5.23, with an increase in the volume of glutaraldehyde and duration of cross-linking, there is a decrease in the rofecoxib release from the microspheres. A burst effect is observed in all the formulations which is attributed to the drug present on the surface of the microspheres. The burst effect could not be reduced even with an increase in the glutaraldehyde volume or duration of crosslinking which indicates that increase in crosslinking density could not avoid the accumulation of the drug at the

surface of the microspheres. With an increase in the gelatin concentration, there is some reduction in the burst release. This indicates that with an increase in the gelatin concentration, more amount of the drug is associated with the microspheres matrix and thus less at the surface. The data obtained from the in-vitro release studies were subjected to model fitting and it can be seen from table 5.26 that the release of rofecoxib from the gelatin microspheres can be best explained by the korsemeyer and peppas model (except batch R-GM7-3 hours and R-GM4 which follows first order release kinetics)as evidenced by the r^2 values. The value of the release exponent 'n' calculated for all the batches is less than 0.5 indicating that the mechanism of rofecoxib release is quasi fickian diffusion. The value of the release rate constant K decreases with an increase in the volume of glutaraldehyde indicating a decrease in the release rate of rofecoxib from the microspheres with an increase in the glutaraldehyde volume. With an increase in the duration of crosslinking in the batch R-GM8 the release rate constant does not significantly decrease and hence it indicates that the duration of crosslinking does not have a significant influence on the release of rofecoxib from the gelatin microspheres. Hence 1 hour was selected as an optimum duration of crosslinking for the preparation of rofecoxib loaded gelatin microspheres. The effect of the different variables on the in-vitro release of the valdecoxib loaded gelatin microspheres is shown in tables 5.27 and 5.28 and figures 5.9 and 5.10. The release kinetic parameters of the microspheres are shown in table 5.30.

Table 5.27: Effect of volume of glutaraldehyde and duration of cross-linking on the drug release from valdecoxib loaded gelatin microspheres

Time(h)	Cumulative % release			
	V-GM5-1 hour (0.5 ml)	V-GM5-3 hours (0.5ml)	V-GM6-1 hour (1.0ml)	V-GM6-3 hours (1.0 ml)
1	43.72±1.84	45.01±1.62	32.14±1.76	35.61±0.69
2	49.06±1.92	48.56±2.51	38.77±1.81	42.3±2.12
3	58.9±2.17	60.24±1.32	43.94±1.73	50.26±1.16
4	67.13±0.98	69.13±2.77	49.5±1.61	57.83±2.00
8	79.24±2.10	77.29±1.26	56.31±1.52	62.73±2.12
24	85.5±2.48	88.63±2.84	62.33±1.33	75.04±0.98
48	94.02±1.62	97.29±1.89	70.05±1.54	87.92±2.45
72			79.61±2.93	95.36±3.01
96			87.18±1.76	
120			98.42±0.79	

Figure 5.9: Effect of volume of glutaraldehyde and duration of cross-linking on the drug release from valdecoxib loaded gelatin microspheres

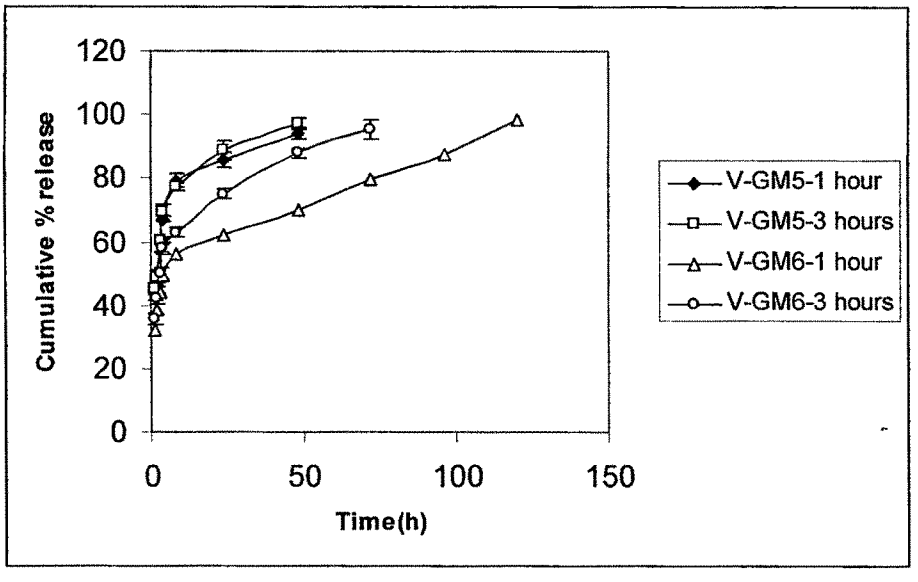


Table 5.28: Effect of gelatin concentration on the release of valdecoxib from gelatin microspheres

Time(h)	Cumulative % release	
	V-GM2 (15%w/w gelatin)	V-GM6 (25% w/w gelatin)
1	45.77±2.07	32.14±1.76
2	53.05±2.37	38.77±1.81
3	60.92±1.74	43.94±1.73
4	69.42±1.59	49.5±1.61
8	75.02±0.91	56.31±1.52
24	87.63±2.58	62.33±1.33
48	95.72±2.23	70.05±1.54
72		79.61±2.93
96		87.18±1.76
120		98.42±0.79

Figure 5.10: Effect of gelatin concentration on the drug release from valdecoxib loaded gelatin microspheres

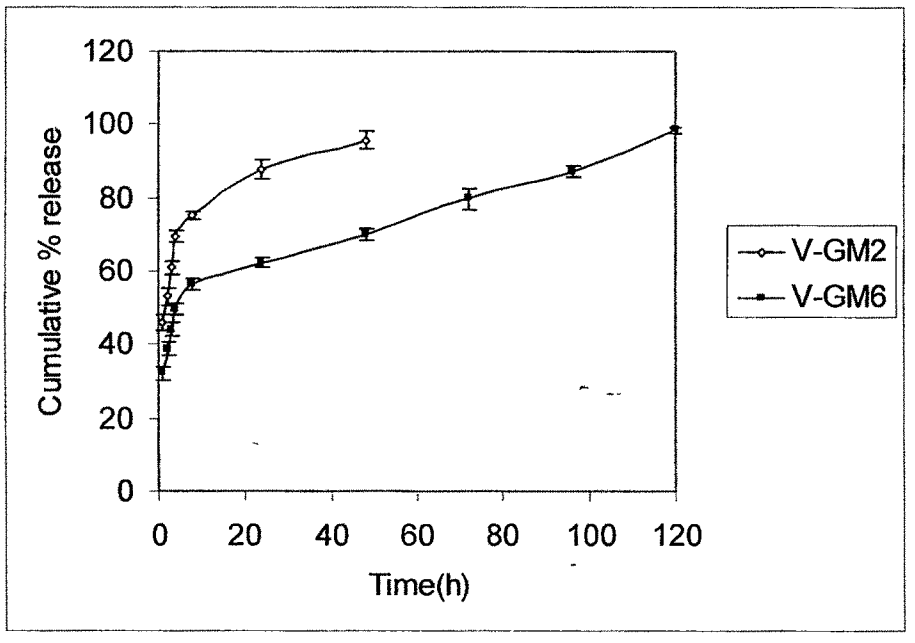


Table 5.29: In-vitro release profile of valdecoxib loaded gelatin microspheres (fitted to Korsemeyer-peppas model)

Log t	Log(Qt/Qα)				
	V-GM5-1 hour	V-GM5-3 hours	V-GM6-1 hour	V-GM6-3 hours	V-GM2
0.000	-0.359	-0.347	-0.492	-0.448	-0.339
0.301	-0.309	-0.314	-0.411	-0.373	-0.275
0.477	-0.230	-0.220	-0.357	-0.298	-0.215
0.602	-0.173	-0.160	-0.305	-0.237	-0.159
0.903	-0.101	-0.112	-0.249	-0.202	-0.125
1.380	-0.068	-0.052	-0.205	-0.124	-0.057
1.681	-0.027	-0.012	-0.154	-0.055	-0.019
1.857			-0.099	-0.020	
1.982			-0.059		
2.079			-0.006		

Table 5.30: Release kinetic parameters of valdecoxib loaded gelatin microspheres

Batch code	Correlation coefficient(r^2)				n (peppas model)	K (peppas model)
	Zero order	Higuchi	First order	peppas		
V-GM5-1 hour	0.696	0.835	0.952	0.922	0.200	0.463
V-GM5-3 hours	0.737	0.868	0.972	0.932	0.204	0.467
V-GM6-1 hour	0.918	0.965	0.853	0.972	0.204	0.342
V-GM6-3 hours	0.846	0.942	0.984	0.971	0.219	0.382
VGM2	0.759	0.887	0.967	0.954	0.187	0.486

n= Release exponent
K= Release rate constant

The release studies of the valdecoxib loaded gelatin microspheres indicated that the drug release from the microspheres prepared using 0.5 ml of glutaraldehyde was not affected by the duration of cross-linking. There was no significant difference in the drug release rates from the microspheres prepared using 1 hour or 3 hours of the duration of cross-linking, in the case where 0.5 ml of glutaraldehyde. In the case

where, 1.0 ml of glutaraldehyde was used, the microsphere cross-linked for 3 hours releases the drug rapidly than the microspheres cross-linked for 1 hour. The reason behind this finding may be the fracture of the microspheres due to prolonged cross-linking. Similar results were also reported by previous workers (Madan et al, 1976).

The release of valdecoxib from the gelatin microspheres can be best explained by first order kinetics (except batch V-GM6-1 hour which follows korsemeyer and peppas model) as evidenced by the r^2 values. The value of the release exponent is less than 0.5 in all the batches, it can be concluded that valdecoxib is released from the gelatin microspheres by quasi-fickian diffusion. The value of release rate constant K decreases with an increase in the glutaraldehyde volume indicating that the release rate decreases with an increase in the cross-linking density of the microspheres. The K value obtained for the microspheres crosslinked for 1 hour is not significantly different from that of the microspheres crosslinked for 3 hours indicating that there is no significant effect of duration of cross-linking on the release rate of valdecoxib from gelatin microspheres.

5.10.2d Scanning electron microscopy studies

The scanning electron microscopy studies revealed that the microspheres had a rough surface with drug crystals on the surface (Figure 5.12). The surface of the plain gelatin microspheres is smooth as shown in figure 5.11.

Figure 5.11: Scanning electron micrograph of plain gelatin microspheres

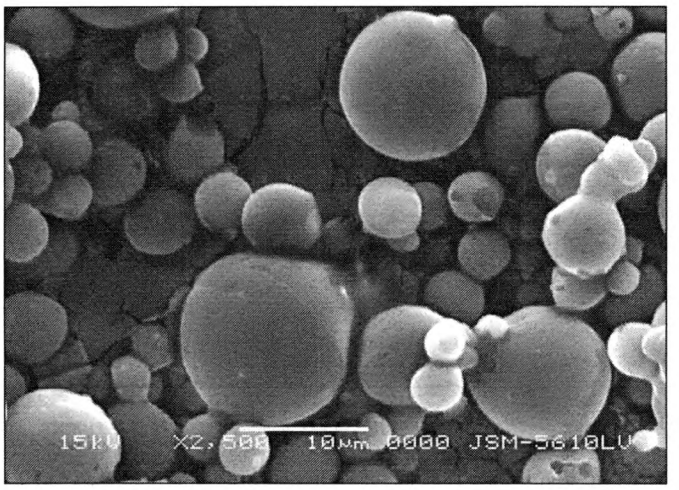
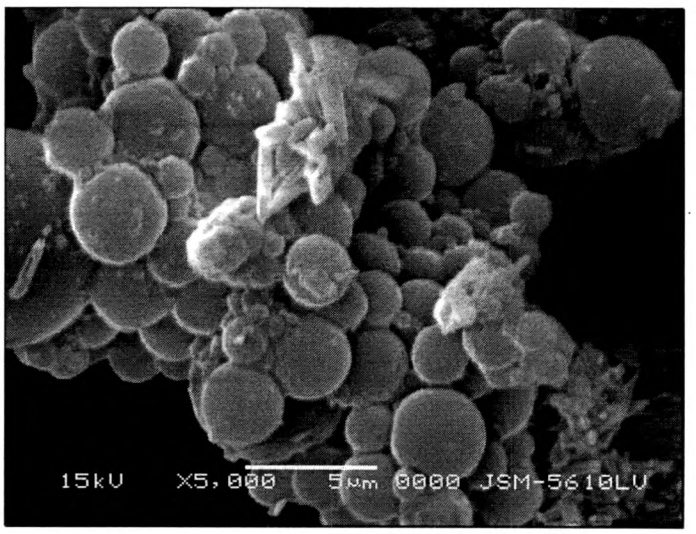


Figure 5.12: Scanning electron micrograph of celecoxib loaded gelatin microspheres



5.10.2e FTIR studies

The FTIR spectrum of gelatin (Figure 5.13) shows the characteristic absorption bands of a protein. The absorption band at 3425.3 cm^{-1} is due to the –N-H stretching, and the band at 1639.4 cm^{-1} is due to the N-H bending. Figure 5.14 shows the FTIR spectrum of Poly-ethylene glycol and Figure 5.15 shows the FTIR spectrum of a mixture of poly-ethylene glycol and gelatin. The mixture of polyethylene glycol and gelatin was prepared by dissolving polyethylene glycol-400 in the gelatin solution and subsequently lyophilizing to get a fine powder. The presence of all the characteristic peaks of both gelatin and poly-ethylene glycol in the FTIR spectrum of the mixture indicates that there is no chemical interaction between gelatin and Poly-ethylene glycol and PEG acts only as a de-aggregating agent in the preparation of the microspheres. The FTIR spectrum of celecoxib is shown in figure 5.16.

The absence of the characteristic absorption band of aldehyde at around 1700 cm^{-1} in the FTIR spectrum of formaldehyde crosslinked microspheres(Figure 5.17) and glutaraldehyde cross-linked microspheres(figure 5.18) indicate the absence of residual free glutaraldehyde and formaldehyde in the crosslinked microspheres.

The presence of an extra absorption band at 1542.9cm^{-1} in the FTIR spectra of both glutaraldehyde and formaldehyde cross-linked microspheres indicate the formation of an imine by reaction of the amine group of gelatin and aldehyde group of the crosslinking agent.

The absence of the characteristic absorption bands of celecoxib in the FTIR spectrum of celecoxib loaded gelatin microspheres confirms that celecoxib is entrapped in the microspheres and is not present as free form.

Figure 5.13: FTIR spectrum of Gelatin

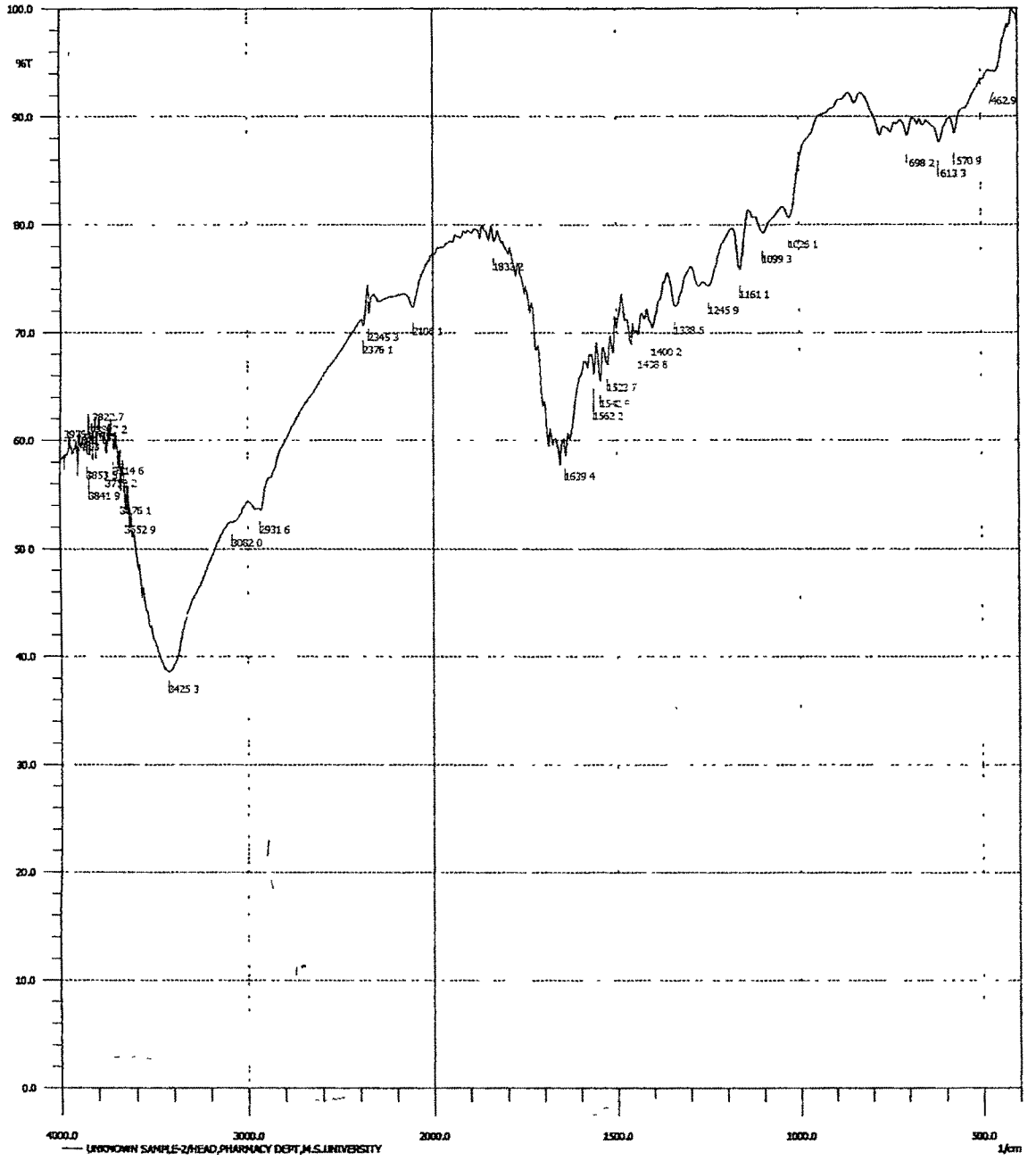


Figure 5.14: FTIR spectrum of poly-ethylene glycol

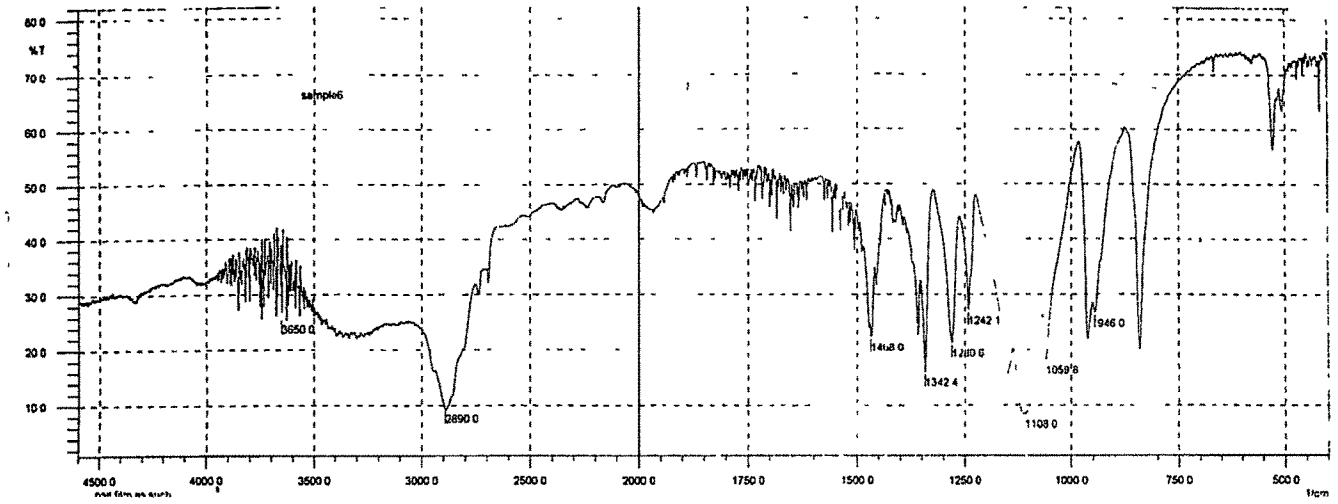


Figure 5.15: FTIR spectrum of a mixture of polyethylene glycol and gelatin

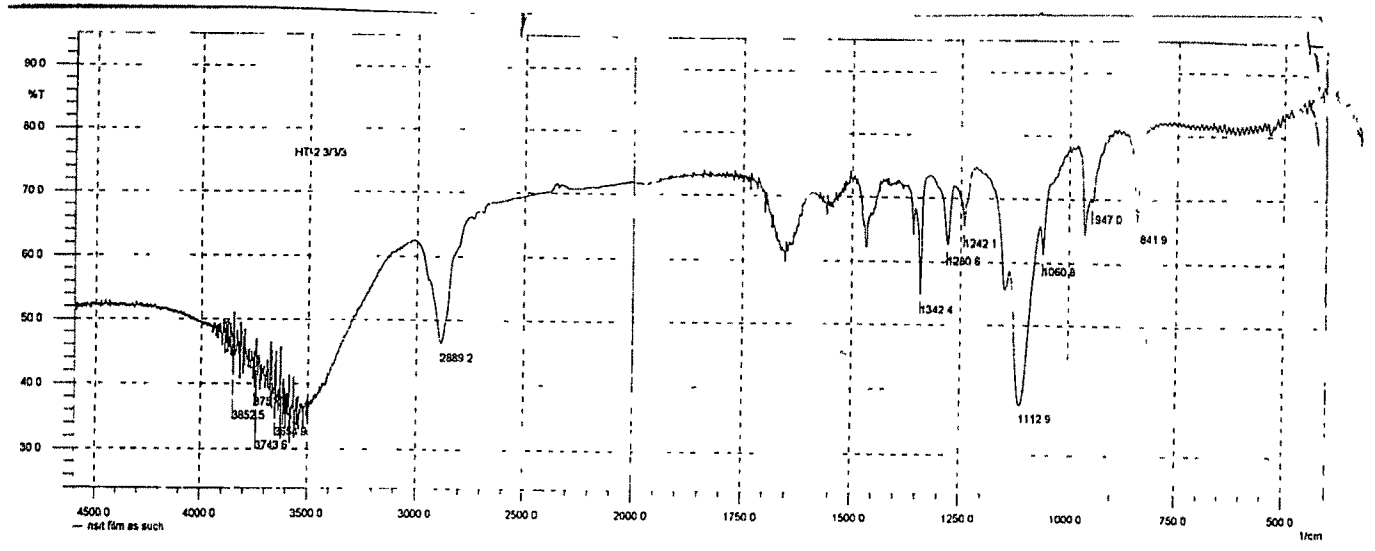


Figure 5.16: FTIR spectrum of celecoxib

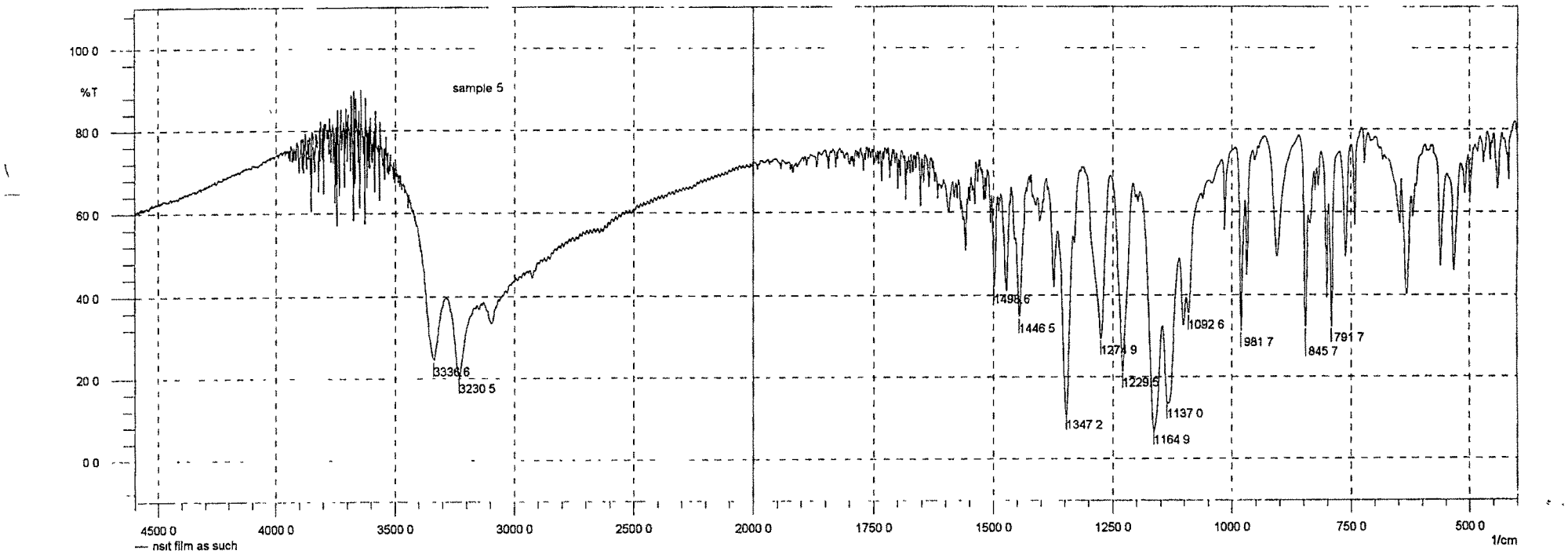


Figure 5.17: FTIR spectrum of formaldehyde crosslinked gelatin microspheres

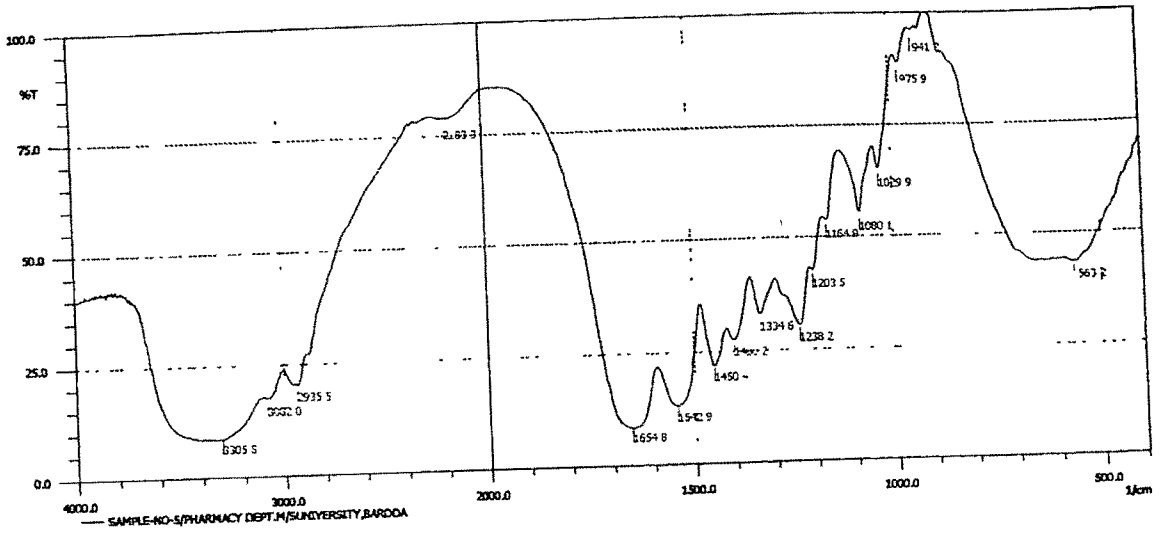
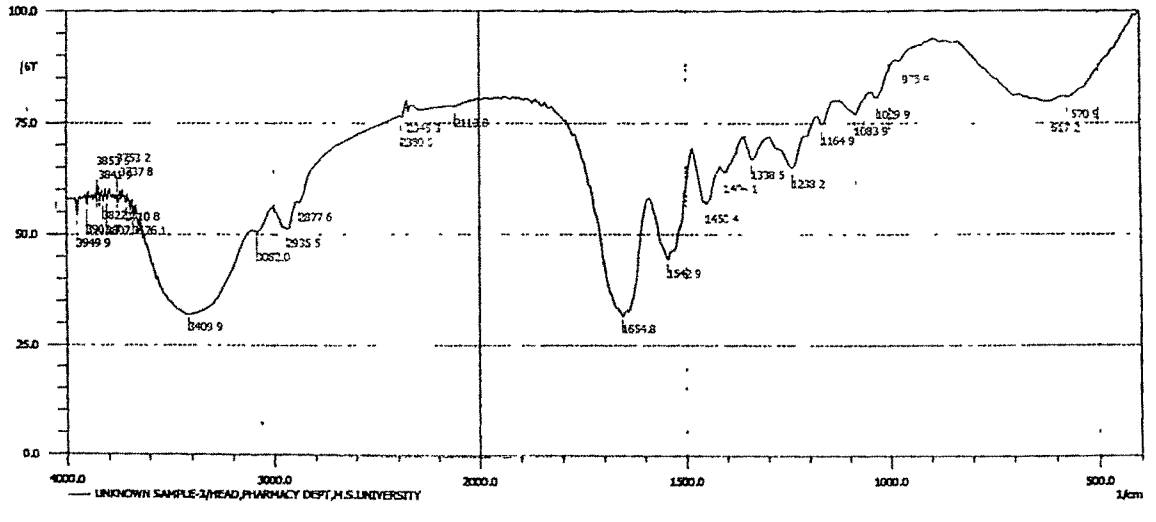


Figure 5.18: FTIR spectrum of glutaraldehyde crosslinked gelatin microspheres



5.10.3 Chitosan microspheres

Due to its bio-compatibility, there have been a substantial number of studies on the biomedical use of chitosan as a drug carrier. Chitosan can be cross-linked to various degrees to modulate drug diffusion in their matrix and hence to achieve a sustained release of drugs. There are numerous reports on the use of glutaraldehyde as a cross-linking agent in the preparation of microspheres (Hassan et al, 1992, Thanoo et al, 1992, Ohya et al, 1993, Akbuga and Durmaz, 1994, Jameela et al, 1998). Very few reports are available where an alternative cross-linking agent/method is described out of which use of formaldehyde or heat (Genta et al, 1997a, Genta et al, 1997b, Lim and Wan, 1995, Kumbhar et al, 2002) for cross-linking chitosan microspheres are prominent. Heat treatment on hydrophilic polymers such as chitosan induces the formation of cross-links between the polymeric molecules and/or formation of crystallites increasing the water resistance of the materials (Genta et al, 1997b). Chitosan microspheres were prepared using three different cross-linking agents viz. glutaraldehyde, formaldehyde and heat. The effect of the cross-linking agents on the characteristics of the microspheres loaded with celecoxib was studied. The effect of various factors on the entrapment efficiency, particle size and drug release characteristics of the microspheres was studied by varying the factor in consideration keeping other variables constant.

5.10.3.1 Entrapment efficiency and particle size

For determining the entrapment efficiency of the microspheres, it was necessary to degrade the microsphere matrix and then determine the drug present in the microspheres. The determination of the entrapped drug by incubating the microspheres with methanol did not extract the drug completely from the microspheres. So, for degrading the microsphere matrix, the microspheres were

incubated with 0.1N Hydrochloric acid for 24 hours. The microspheres did not completely dissolve in 0.1N Hydrochloric acid, but the degradation of the microsphere matrix was confirmed by microscopy. The drug was then extracted from this dispersion using dichloromethane. The organic extract was then evaporated to dryness and the residue dissolved in methanol. Absorbance of this solution was then measured using methanol as blank. The entrapment efficiency was then calculated as shown in section 5.4.

The effect of the different variables on the entrapment efficiency and the particle size was as follows:

5.10.3.1a Effect of cross-linking agent

The effect of the three different cross-linking agents viz. glutaraldehyde, formaldehyde and heat on the entrapment efficiency and the particle size was studied.

The glutaraldehyde cross-linked microspheres were yellowish brown in colour and were obtained as a fine powder. As shown in table 5.31, the glutaraldehyde volume used for cross-linking and the duration of cross-linking does not have significant influence ($p>0.1$) on the entrapment efficiency and the particle size of the microspheres. The microspheres cross-linked using glutaraldehyde shows high entrapment efficiency of around 90%.

The formaldehyde cross-linked microspheres were obtained as a white fine powder. As shown in table 5.32, the volume of formaldehyde and the duration of cross-linking does not have a significant influence ($p>0.1$) on the entrapment efficiency and the particle size of the microspheres. The formaldehyde cross-linked microspheres shows high entrapment efficiency of around 90%.

In case of heat crosslinked microspheres, no microspheres were formed at the temperature of 60°C (table 5.33). A temperature of 60°C may not be sufficient for complete evaporation of water from the internal phase of the emulsion. Thus a jelly like material was obtained instead of microspheres. The heat cross-linked microspheres prepared at 70°C or 90°C were obtained as a fine white powder. The heat cross linked microspheres shows significantly lower ($p<0.05$) entrapment efficiency than the chemically cross-linked microspheres. As shown in table 5.33, both batches I and J show the entrapment efficiency of only 20-25%. The temperature at which the heat cross-linked microspheres are prepared has an influence on the entrapment efficiency. Microspheres prepared at 70°C shows higher entrapment efficiency than the microspheres prepared at 90°C. The reason behind this finding is that the solubility of celecoxib in the external phase of the w/o emulsion increases with an increase in the temperature.

The microspheres were prepared by emulsion polymerization technique. In this method, the drug is dispersed in the chitosan solution and then emulsified in the oily phase in presence of an emulsifier. Then the chitosan present in the internal phase is cross-linked either by chemical or thermal treatment. Since celecoxib is not soluble in the external phase of the emulsion and in the crosslinking agents glutaraldehyde or formaldehyde at the conditions used for preparation of microspheres, higher entrapment efficiency was obtained for chemically cross-linked microspheres. In case of heat cross-linking, the higher temperature used for the preparation of the microspheres solubilizes the drug in the external phase of the emulsion and the drug migrates to the external phase. Thus very less entrapment efficiency of around 20%-25% was obtained in case of heat cross-linked

microspheres. This was confirmed by the presence of the solubilized celecoxib in the external phase of the emulsion.

The particle size of the microspheres was affected by the cross-linking agent used. The formaldehyde cross-linked microspheres shows a larger particle size than the glutaraldehyde cross-linked microspheres and the lowest particle size was obtained for the heat cross-linked microspheres. Crosslinking with glutaraldehyde is reported to produce greater number and more stable cross-links than with formaldehyde (Oppenheim, 1987). This may be the reason of the smaller particle size obtained for the glutaraldehyde cross-linked microspheres. The heat cross-linked microspheres exhibit the smallest particle size. The geometric mean diameter of the heat cross-linked microspheres decreased from 9.62 to 6.48 as the temperature was increased from 70°C to 90°C.

The particle size of the heat cross-linked microsphere is significantly smaller than the chemically cross-linked microspheres ($p<0.05$). Similar results were obtained by previous workers (Genta et al, 1997b, Kumbhar et al, 2002). This may be because of the fact, that there is complete dehydration of the microspheres resulting in the shrinkage at the temperature at which the microspheres are prepared.

Thus from the results obtained for the different cross-linking agents, glutaraldehyde seems to be most promising as a cross-linking agent, as the glutaraldehyde cross-linked microspheres have higher entrapment efficiency and lower particle size.

Table 5.31: Effect of volume of glutaraldehyde (25%w/w) and duration of cross-linking on the entrapment efficiency and particle size of celecoxib loaded chitosan microspheres

Batch code	Volume of glutaraldehyde (ml)	Duration of cross-linking (h)	% Entrapment efficiency	Particle size (μm)
A	0.5	1	89.16±1.32	10.53±0.77
B	0.5	3	88.54±1.65	9.13±0.37
C	1.0	1	89.75±1.75	10.55±1.30
D	1.0	3	90.14±1.60	8.65±1.59

Table 5.32: Effect of volume of formaldehyde (37%w/w) and duration of cross-linking on the entrapment efficiency and particle size of celecoxib loaded chitosan microspheres

Batch code	Volume of formaldehyde	Duration of cross-linking(h)	% Entrapment efficiency	Particle size(μm)
E	0.5 ml	1	88.72±1.71	14.68±0.98
F	0.5 ml	3	90.15±1.59	12.60±1.18
G	1.0 ml	1	91.64±3.69	11.62±1.12
H	1.0 ml	3	90.53±1.69	12.75±1.08

Table 5.33: Effect of temperature on entrapment efficiency and particle size of the celecoxib loaded heat cross-linked microspheres

Batch code	Temperature (° C)	% Entrapment efficiency	Particle size (μm)
I	60	No microspheres were formed	
J	70	24.56±0.78	9.62±1.22
K	90	20.91±2.50	6.48±0.67

5.10.3.1b Effect of stirring speed

To study the effect of stirring speed on the entrapment efficiency and particle size, the microspheres were prepared using three different stirring speeds viz. 1500 rpm, 2500 rpm and 4000 rpm. As shown in table 5.34, with an increase in the stirring speed from 1500 rpm to 2500 rpm, there was a decrease in the particle size from 12.68μm to 8.65μm. With further increase in the stirring speed to 4000 rpm, there

was no significant decrease in the particle size. Thus, 2500 rpm was selected as an optimum stirring speed. The stirring speed does not have any influence on the entrapment efficiency of the microspheres.

Table 5.34: Effect of stirring speed on the entrapment efficiency and particle size of celecoxib loaded chitosan microspheres

Stirring speed (rpm)	% Entrapment efficiency	Particle size (µm)
1500	91.86±1.22	12.68±0.85
2500	90.14±1.60	8.65±1.59
4000	88.56±0.96	7.92±0.27

Chitosan concentration: 3%w/w
Span-85 concentration: 5%w/w

5.10.3.1c Effect of volume ratio of water: oil phase

Three different ratios of volume of water: oil phase, 1:10, 1:20 and 1:40. The microspheres were aggregated in case of 1:10 ratio and were found to be discrete in case of 1:20 and 1:40. Thus, 1:20 was selected as an optimum ratio of volume of water: oil phase. The particle size of the 1:10 was also significantly higher than the 1:20 and 1:40 ratio. There was no significant difference in the entrapment efficiency of the batches prepared using different ratios.

5.10.3.1d Effect of composition of external phase

Three different compositions of external phase were used to study the effect of the parameter on the entrapment efficiency and the particle size. Light liquid paraffin, heavy liquid paraffin and a mixture of light and heavy liquid paraffin (1:1) were used. As shown in table 5.35, the geometric mean diameter of the microspheres prepared using light liquid paraffin and a mixture of light and heavy liquid paraffin was significantly higher than that of the microspheres prepared using heavy liquid paraffin. The entrapment efficiency of the microspheres was not significantly affected by the composition of the external phase.

Table 5.35: Effect of composition of external phase on the entrapment efficiency and particle size of celecoxib loaded chitosan microspheres

External phase	% Entrapment efficiency	Particle size (μm)
Light liquid paraffin	93.27±2.14	16.89±1.38
Heavy liquid paraffin	90.14±1.60	8.65±1.59
Light:Heavy liquid paraffin(1:1)	91.09±1.83	12.81±1.25

5.10.3.1d Effect of chitosan concentration, span-85 concentration and volume of glutaraldehyde

A 2^3 factorial design was used to investigate the combined effect of three different variables in the preparation of celecoxib loaded chitosan microspheres. The concentration of gelatin, concentration of span-85 and volume of glutaraldehyde were selected as causal factors while the particle size and the % entrapment efficiency were selected as the dependent variables. Two levels, low and high were selected for all the three factors. Potential variables such as the stirring speed, concentration of tween-80, volume ratio of oil: water phase, temperature of the external phase and emulsification time were kept constant in the experimental design. Based on the factorial design, eight batches of celecoxib loaded chitosan microspheres were prepared as shown in table 5.36. The main and the interaction effects of the variables on the entrapment efficiency and the particle size of the microspheres were studied. Mathematical modeling was carried out to obtain a polynomial equation. (full model, equation 1)(Anthony et al, 1996).

Table 5.36: Optimization of parameters for preparation of celecoxib loaded chitosan microspheres

Batch No.	Concentration of chitosan (%w/w)	Concentration of span-85 (%w/w)	Volume of glutaraldehyde (ml)	% Entrapment efficiency	Particle size (μm)
CMS-1	2	2	0.5	72.1	11.64
CMS-2	2	2	1.0	73.18	12.85
CMS-3	2	5	0.5	62.29	9.05
CMS-4	2	5	1.0	65.32	9.13
CMS-5	3	2	0.5	94.24	16.65
CMS-6	3	2	1.0	95.38	15.95
CMS-7	3	5	0.5	88.54	9.13
CMS-8	3	5	1.0	90.14	8.65

Table 5.37: Coded values of the formulation parameters of celecoxib loaded chitosan microsphere by 2^3 factorial design

Coded values	Actual values		
	X1	X2	X3
-1	2	2	0.5
+1	3	5	1.0

X1 = Concentration of chitosan (%w/w)
X2 = Concentration of span-85 (%w/w)
X3 = Volume of glutaraldehyde (ml)

Table 5.38: 2^3 Factorial design layout of celecoxib loaded chitosan microspheres

Batch no.	X1	X2	X3	X1X2	X2X3	X1X3	% Entrapment efficiency	Particle size (μm)
1	-1	-1	-1	1	1	1	72.1	11.64
2	-1	-1	1	1	-1	-1	73.18	12.85
3	-1	1	-1	-1	-1	1	62.29	9.05
4	-1	1	1	-1	1	-1	65.32	9.13
5	1	-1	-1	-1	1	-1	94.24	16.65
6	1	-1	1	-1	-1	1	95.38	15.95
7	1	1	-1	1	-1	-1	88.54	9.13
8	1	1	1	1	1	1	90.14	8.65

Table 5.39: Model coefficients estimated by multiple linear regression for celecoxib loaded chitosan microspheres by 2^3 factorial design (Entrapment efficiency)

Factor	Coefficients	Computed t-value	p-value*
Intercept	80.148	430.328	0.0014
X1	11.926	64.033	0.0099
X2	-3.576	-19.201	0.033
X3	0.856	4.597	0.136
X1X2	0.841	4.516	0.138
X2X3	0.301	1.617	0.352
X1X3	-0.171	-0.919	0.526

*Significant at $p<0.05$

Table 5.40: Analysis of variance (ANOVA) of full and reduced models of celecoxib loaded chitosan microspheres by 2^3 factorial design (Entrapment efficiency)

		DF	SS	MS	F	R	R^2	Adj R^2
Regression	FM	6	1252.68	208.78	752.33	0.999	0.999	0.998
	RM	2	1240.2	620.1	242.88	0.994	0.989	0.985
Error	FM	1	0.277(E1)	0.277	752.33			
	RM	5	12.765(E2)	2.553	242.88			

SSE2-SSE1=12.765-0.277= 12.488
No.of parameters omitted = 4
MS of error (full model) =0.277
F calculated = (12.488/4)/0.277=11.27

Table 5.41: Model coefficients estimated by multiple linear regression for celecoxib loaded chitosan microspheres by 2^3 factorial design (particle size)

Factor	Coefficients	Computed t-value	p-value*
Intercept	11.631	68.925	0.009
X1	0.963	5.711	0.110
X2	-2.64	-15.651	0.040
X3	0.013	0.081	0.948
X1X2	-1.063	-6.303	0.100
X2X3	-0.113	-0.674	0.622
X1X3	-0.308	-1.829	0.318

*Significant at $p \leq 0.1$

Table 5.42: Analysis of variance (ANOVA) of full and reduced models of celecoxib loaded chitosan microspheres by 2^3 factorial design (Particle size)

		DF	SS	MS	F	R	R^2	Adj R^2
Regression	FM	6	73.160	12.193	53.523	0.998	0.996	0.978
	RM	2	64.862	32.431	19.019	0.940	0.883	0.837
Error	FM	1	0.227(E1)	0.227	53.523			
	RM	5	8.52(E2)	1.705	19.019			

SSE2-SSE1=8.52-0.227=8.293
No. of parameters omitted=4
MS of error (full model) = 0.227
F calculated = (8.293/4)/0.227=9.133

The % entrapment efficiency obtained at various levels of three independent variables (X1, X2 and X3) was subjected to multiple linear regression to yield a second order polynomial equation (full model. Equation 5)

$$Y=80.14+11.92X1-3.576X2+0.856X3+0.841X1X2+ 0.301X2X3-0.171X1X3 \quad \text{(Equation 5)}$$

The main effects of X1, X2 and X3 represent the average result of changing one variable at a time from its low to high value. The interactions(X1X2, X2X3 and X1X3) show how the entrapment efficiency changes when two or more variables are simultaneously changed. The entrapment efficiency for the eight batches shows a wide variation from 65.32% to 95.38%. Small values of coefficients in terms of X3, X1X2, X2X3 and X1X3 are regarded as least contributing in the preparation of celecoxib loaded gelatin microspheres. Hence these non-significant terms are neglected from the full model and a reduced polynomial equation (equation 6) was obtained following multiple regression of entrapment efficiency and very significant ($p<0.05$) terms of equation 5.

$$Y= 80.14+11.92X1-3.57X2 \quad \text{(Equation 6)}$$

The significance of each coefficient of the equation 1 was determined by student't' test and p-value which showed that the quadratic main effects of the concentration of chitosan (p value= 0.0099) and concentration of span-85($p=0.033$) are found to be extremely significant. The interaction between the different variables is not significant as evidenced from their p values.

ANOVA between the full and the reduced model was performed. F-statistic of the results of the full and reduced model confirmed omission of non-significant terms of equation 5. When the coefficients of the four independent variables in equation 1 were compared, the value of the variable X1($b1=11.92$) and X2($b2=3.57$) was found to be

maximum and hence both the variables, concentration of chitosan and concentration of span-85 were considered to be major contributing variables for entrapment efficiency of celecoxib microspheres. The goodness of fit of the model was checked by the determination of coefficient (R^2). In this case, the values of the determination coefficients (R^2 =0.999 for the full model and 0.989 for the reduced model) indicated that over 90% of the total variations are explained by the model. The values of adjusted determination coefficients (adj R^2 = 0.998 for full model and 0.985 for reduced model) are also very high which indicates a high significance of the model. All the above considerations indicate and excellent adequacy of the regression model (Adinarayana et al, 2002, Box et al, 1978, Cochran and Cox, 1992).

The particle size obtained for the various batches was subjected to multiple linear regression to yield a polynomial equation (equation 7, full model)

Y=11.631+0.963X1-2.64X2+0.013X3-1.063X1X2-

0.113X2X3-0.308X1X3 .....(Equation 7)

The geometric mean diameter of the eight batches shows a variation from 8.65μm to 16.65 μm. Small values of coefficients in terms of X1, X3, X1X3 and X1X4 are regarded as least contributing in the preparation of celecoxib loaded gelatin microspheres. Hence these non-significant terms are neglected from the full model and a reduced polynomial equation (equation 8) was obtained following multiple regression of entrapment efficiency and very significant ($p<0.05$) terms of equation 7.

Y= 11.631 – 2.64X2-1.063X1X2.....(Equation 8)

The significance of each coefficient of the equation 7 was determined by student't' test and p-value which showed that the quadratic main effects of the concentration of span-85 (p value=0.040 is found to be significant. There is an interaction between the variables X1, concentration of gelatin and X2, Concentration of span-85. There is a

combined influence of both the parameters on the particle size of the microspheres (p=0.100).

ANOVA between the full and the reduced model was performed. F-statistic of the results of the Full and reduced model confirmed omission of non-significant terms of equation 7. When the coefficients of the three independent variables in equation 7 were compared, the value of the variable X2(b1=2.64), X1X2(b4=1.063) was found to be maximum and hence the variables concentration of span-85, and concentration of chitosan were considered to be major contributing variables for particle size of chitosan microspheres. The goodness of fit of the model was checked by the determination of coefficient (R^2). In this case, the values of the determination coefficients R^2=0.996 for the full model and 0.940 for the reduced model indicated that over 90% of the total variations are explained by the model. The above considerations indicate and excellent adequacy of the regression model (Adinarayana et al, 2002, Box et al, 1978, Cochran and Cox, 1992).

Thus, from the results of the factorial design, it is evident that the main factors affecting the entrapment efficiency of chitosan microspheres are the concentration of chitosan and concentration of span-85. With an increase in the chitosan concentration from 2%w/w to 3%w/w, there is an increase in the entrapment efficiency from around 72% to 97%. This increase in the entrapment efficiency is because of the increase in the viscosity of the chitosan solution with an increase in its concentration. With an increase in the viscosity, more amount of the drug is associated with the microspheres and less amount is present as free drug. There is a decrease in the entrapment efficiency with an increase in the span-85 concentration. This is due to the solubility of celecoxib in the external phase of the emulsion at higher concentration of span-85. This effect is more pronounced at lower concentration of chitosan, which indicates

that the less viscous solutions of chitosan can less efficiently prevent the dissolution of the drug in the external phase.

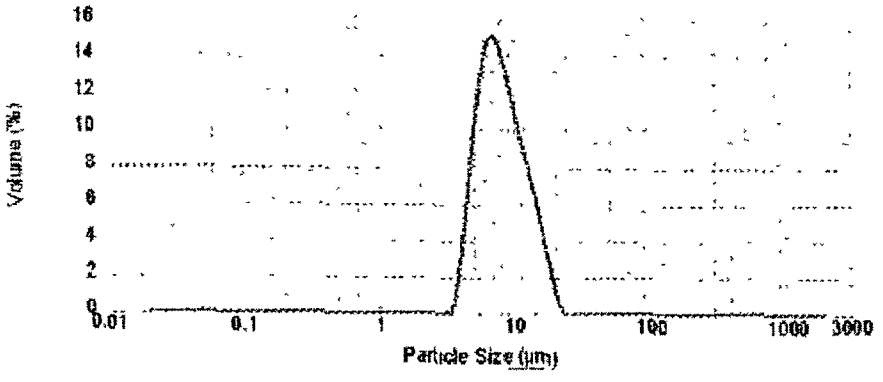
The main variable affecting the particle size of the microspheres is the concentration of span-85. With an increase in the emulsifier concentration, the interfacial tension between the aqueous and the oil phase decreases and hence the particle size decreases. The volume of glutaraldehyde does not have a significant influence on the entrapment efficiency or the particle size of the chitosan microspheres.

There is a combined effect of the concentration of chitosan and concentration of span-85 on the particle size of the microspheres. At higher concentration of chitosan, the effect of span-85 on the particle size was found to be more prominent.

Thus batch containing 3% chitosan, 5% span-85 and 1ml glutaraldehyde which gave the lowest particle size of less than 10μm and high entrapment efficiency of 90.14% was selected as an optimized batch.

The particle size distribution of the optimized batch of celecoxib loaded chitosan microspheres as shown in figure 5.19 shows that the particle size is monodisperse.

Figure 5.19: Particle size distribution of celecoxib loaded chitosan microspheres



The results obtained for celecoxib loaded chitosan microspheres indicate that glutaraldehyde cross-linking gave the microspheres with desired properties of high

entrapment efficiency and low particle size. Also, for glutaraldehyde cross-linked microspheres, the main factors affecting the entrapment efficiency and the particle size of the microspheres were the concentration of chitosan and the concentration of span-85. Hence, for the preparation of rofecoxib and valdecoxib a loaded chitosan microsphere, the effect of changing these two variables on the entrapment efficiency and particle size was studied. Also the glutaraldehyde content was varied in order to study the effect of glutaraldehyde volume on the entrapment efficiency, particle size and drug release. The entrapment efficiencies and particle size of the various batches prepared for rofecoxib and valdecoxib loaded chitosan microsphere are depicted in tables 5.43 and 5.44 respectively.

Table 5.43: Effect of chitosan concentration on span-85 concentration on the entrapment efficiency and particle size of rofecoxib loaded chitosan microspheres

Batch code	Concentration of chitosan (%w/w)	Concentration of span-85 (%w/w)	Volume of glutaraldehyde (ml)	% Entrapment efficiency	Particle size (μm)
R-CM1	2	2	0.5	83.58±1.52	11.75±2.70
R-CM2	2	5	0.5	81.40±2.55	8.90±1.15
R-CM3	2	7.5	0.5	80.31±2.96	10.37±2.81
R-CM4	2	5	1.0	No microspheres were formed- jelly like material obtained on the filter	
R-CM5	3	5	0.5	No microspheres were formed-hard aggregates were obtained	
R-CM6	3	5	1.0	No microspheres were formed- a jelly like material obtained on the filter	
R-CM7	3	5	0.1	92.30±3.25	18.84±1.87
R-CM8	3	5	0.25	90.72±2.76	15.07±1.85

Table 5.43 shows the particle size and entrapment efficiency of the rofecoxib loaded chitosan microspheres, it can be seen there is no effect of concentration of span-85 on

the entrapment efficiency of the microspheres. There is a significant effect of the concentration of span-85 on the particle size of the microspheres. Since, lowest particle size was obtained for the microspheres prepared using 5% span-85 concentration; it was selected as an optimum concentration for the preparation of the microspheres.

At the chitosan concentration of 2%w/w, as the volume of glutaraldehyde was increased from 0.5 ml to 1.0 ml, no microspheres could be formed. The reason behind this may be the interparticle cross-linking between the chitosan microdrops at higher volumes of glutaraldehyde leading to agglomeration of the microspheres.

At the chitosan concentration of 3%w/w, no microspheres could be formed even with 0.5ml glutaraldehyde. Discrete microspheres were formed with 0.25 ml of glutaraldehyde. At a glutaraldehyde volume of 0.5ml, instead of microspheres, hard aggregates were obtained which could not be disintegrated. This indicates that with an increase in the crosslinking agent, intraparticle crosslinking occurs in addition to interparticle crosslinking leading to agglomeration of the microspheres and formation of hard aggregates. With further increase in the volume of glutaraldehyde to 1.0 ml, a thin jelly like mass was obtained on the filter which could not be recovered in the form of microspheres. This indicates extensive cross-linking between the microdrops of the w/o emulsion.

At a chitosan concentration of 3%w/w, microspheres were formulated using 0.25 ml and 0.1 ml of glutaraldehyde. There was no significant difference between the entrapment efficiency of the microspheres prepared using 0.25 ml or 0.1 ml of glutaraldehyde. High entrapment efficiency of around 90% was obtained for rofecoxib loaded chitosan microspheres. The particle size of the microspheres

prepared using 0.1 ml of glutaraldehyde was higher than that of the microspheres prepared using 0.25 ml of glutaraldehyde.

The effect of the different variables on the entrapment efficiency and particle size of the valdecoxib loaded chitosan microspheres is shown in table 5.44.

Table 5.44: Effect of chitosan concentration, span-85 concentration and volume of glutaraldehyde on the particle size and entrapment efficiency of valdecoxib loaded chitosan microspheres

Batch code	Concentration of chitosan (%w/w)	Concentration of span-85 (%w/w)	Volume of glutaraldehyde (ml)	% Entrapment efficiency	Particle size (μm)
V-CM1	2	2	0.5	75.29±1.64	8.16±0.29
V-CM2	2	5	0.5	68.47±2.17	7.05±0.12
V-CM3	2	7.5	0.5	60.50±3.26	8.42±0.60
V-CM4	3	2	0.5	92.46±2.09	12.84±0.58
V-CM6	3	5	0.5	88.71±2.77	9.69±0.96
V-CM7	3	5	1.0	87.48±2.74	9.06±0.66
V-CM8	3	5	1.5	88.60±2.29	8.88±0.79

Table 5.44 shows that the chitosan concentration has a significant influence on the entrapment efficiency of valdecoxib loaded chitosan microspheres. The entrapment efficiency of batches prepared using 3% chitosan is significantly higher ($p<0.05$) than those prepared using 2% chitosan.

The concentration of span-85 has a significant influence on the entrapment efficiency of the microspheres. At 2% concentration of chitosan, the effect of span-85 on the entrapment efficiency is highly significant. There is a steady decrease in the entrapment efficiency as the concentration of span-85 is increased from 2% to 7.5%. The effect of concentration of span-85 on the entrapment efficiency is less prominent at 3% chitosan.

The chitosan concentration has a significant influence on the particle size of the microspheres. The geometric mean diameter of the microspheres prepared using 3% is significantly higher than those prepared using 2% chitosan. The concentration of span-85 has a significant influence on the particle size. When concentration of span-85 is increased from 2% to 5% there is a decrease in the particle size. With further increase in the concentration of span-85 to 7.5% there is no significant decrease in the particle size.

The volume of glutaraldehyde has no significant influence on the entrapment efficiency or the particle size of the microspheres.

5.10.3.2 Drug release

The drug release from the microspheres was dependent on the cross-linking agent used. The effect of the different variables on the release of celecoxib from chitosan microspheres is shown in tables 5.45 to 5.49 and figures 5.20 to 5.25. The release kinetic parameters of the celecoxib loaded chitosan microspheres are shown in table 5.51.

Table 5.45: Effect of glutaraldehyde volume and duration of cross-linking on the release of celecoxib from chitosan microspheres

Time (h)	Cumulative % release			
	Batch A (0.5ml-1 hour)	Batch B (0.5 ml-3 hours)	Batch C (1.0 ml-1 hour)	Batch D (1.0 ml-3 hours)
1	65.58±1.40	57.9±2.54	53.32±2.76	50.72±2.11
2	68.82±1.49	59.8±1.85	57.86±2.15	54.69±1.84
4	75.68±1.28	77.35±1.72	60.58±0.98	54.75±1.01
8	81.02±2.66	80.72±1.42	65.73±2.57	60.73±2.34
24	88.08±2.29	83.47±1.88	72.12±1.80	64.55±2.88
48	96.72±1.37	84.51±2.17	78.56±2.82	67.51±1.32
72		95.95±3.02	83.18±2.70	75.64±2.68
96		97.73±2.11	95.63±3.59	85.61±2.89

Figure 5.20: Effect of volume of glutaraldehyde (25%w/v) and duration of cross-linking on release of celecoxib from chitosan microspheres

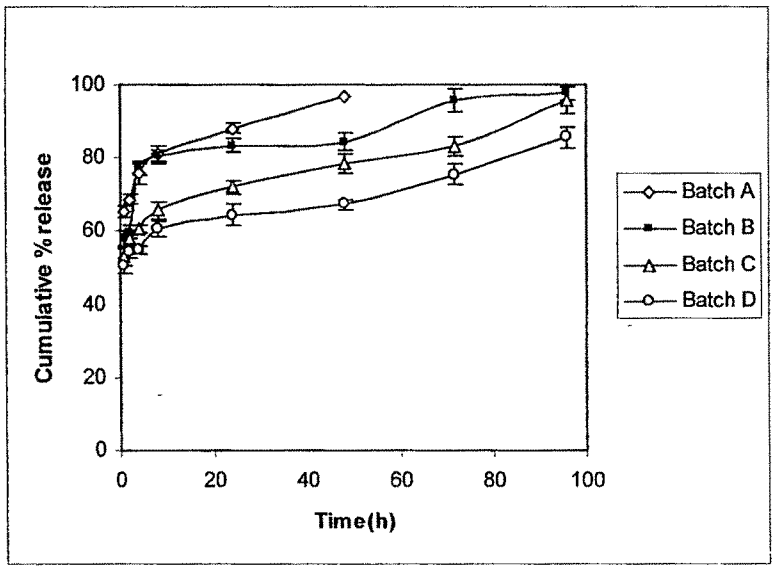


Table 5.46: Effect of volume of formaldehyde and duration of cross-linking on the release of celecoxib from chitosan microspheres

Time (h)	Cumulative % release			
	Batch E (0.5 ml-1 hour)	Batch F (0.5 ml- 3 hours)	Batch G (1.0 ml-1 hour)	Batch H (1.0 ml-3 hours)
1	65.78±2.06	60.51±2.59	54.26±1.76	53.85±1.76
2	68.02±2.48	65.44±3.10	60.72±1.87	58.93±2.91
4	75.91±2.58	68.85±3.61	65.58±2.83	62.07±3.86
8	82. 7±2.35	70.13±3.60	69.01±3 38	65.78±2.37
24	90.61±3.48	80.5±2.96	75.55±1.63	73.84±3.43
48	97.35±3.34	88.92±2 21	89.6±2.24	84.19±2.81
72		95.71±2.32	96.04±1.86	90.47±2.61
96				

Figure 5.21: Effect of volume of formaldehyde (37%w/v) and duration of Cross-linking on the release of celecoxib from chitosan microspheres

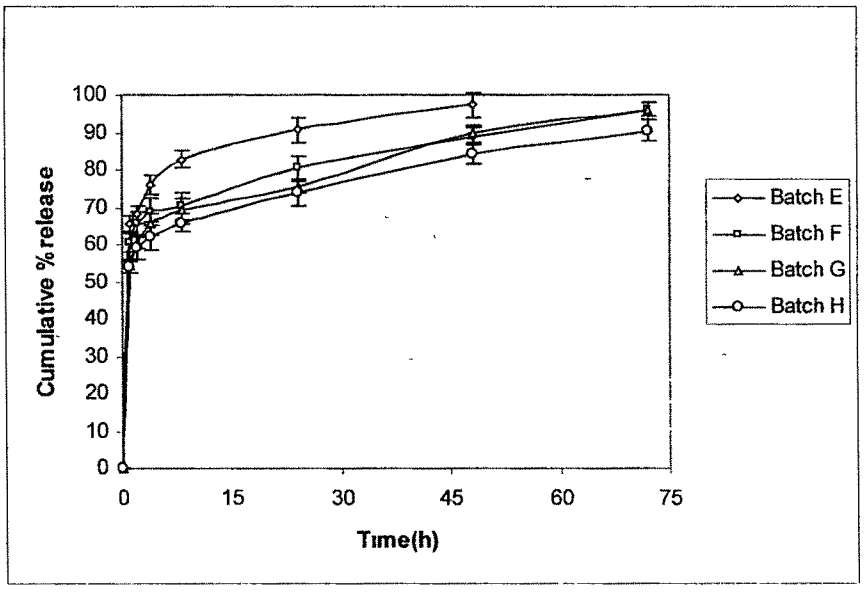


Table 5.47: Effect of temperature on the drug release of celecoxib from chitosan microspheres

Time(h)	Cumulative % release	
	Batch J (70°C)	Batch K (90°C)
1	70.83±2.29	73.57±2.91
2	81.05±3.13	86.05±2.16
4	90.61±2.51	92.38±1.11
8	95.64±3.36	96.4±1.49

Figure 5.22: In-vitro release profile of celecoxib loaded heat cross-linked microspheres

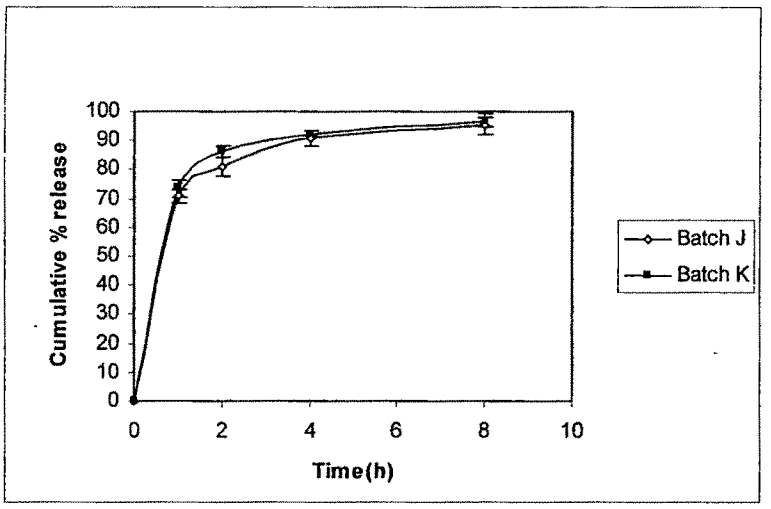


Figure 5.23: Comparative drug release profiles of glutaraldehyde (Batch D), Formaldehyde (Batch H) and heat cross-linked (Batch K) microspheres

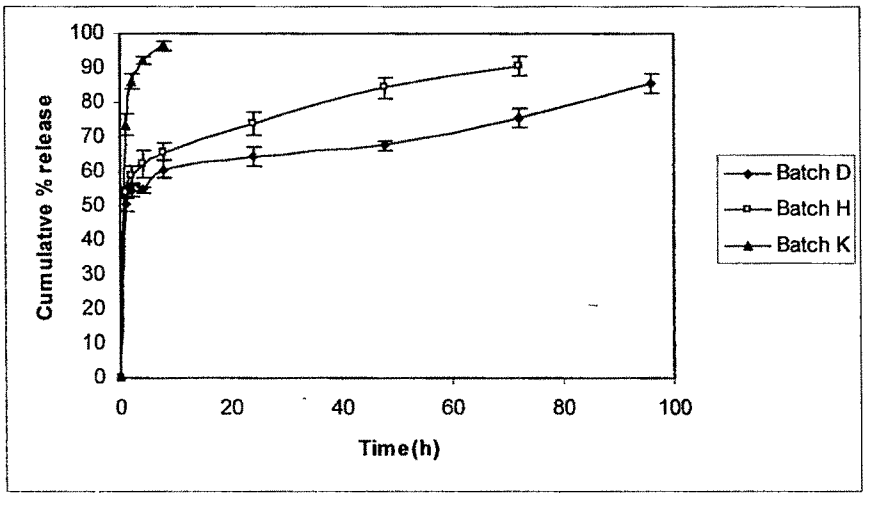


Table 5.48: Effect of chitosan concentration on release of celecoxib from chitosan microspheres

Time(h)	Cumulative % release	
	Batch CMS-4 (2%w/w)	Batch D (3%w/w)
1	65.41±2.86	50.72±2.11
2	69.57±2.51	54.69±1.84
4	75.36±1.86	54.75±1.01
8	80.66±3.14	60.73±2.34
24	88.54±3.59	64.55±2.88
48	95.31±1.58	67.51±1.32
72	98.83±0.77	75.64±2.68
96		85.61±2.89

Figure 5.24: Effect of chitosan concentration on the release of celecoxib from chitosan microspheres

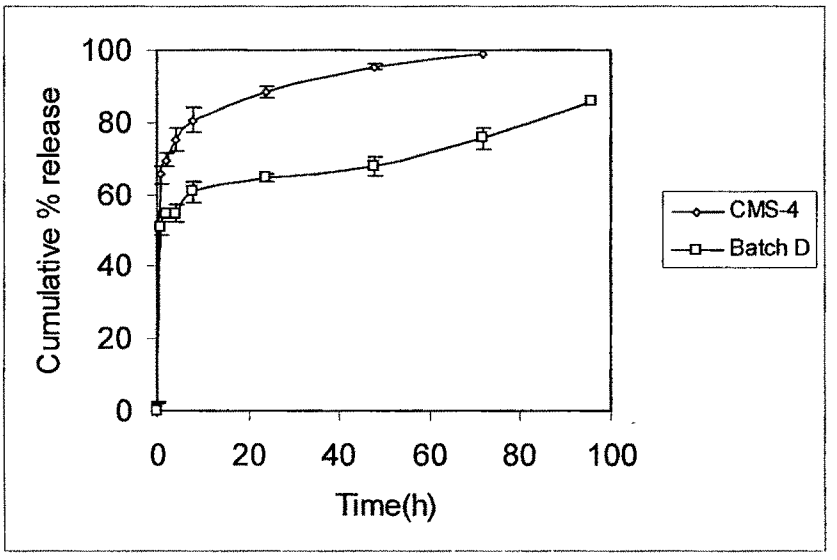


Table 5.49: Effect of presence of collagenase in the dissolution medium on the release of celecoxib from chitosan microspheres

Time(h)	Cumulative % release of celecoxib from chitosan microspheres (Batch D)	
	Without collagenase	With collagenase
1	50.72±2.11	55.82±2.34
2	54.69±1.84	62.4±1.64
4	54.75±1.01	70.69±1.74
8	60.73±2.34	75.62±1.29
24	64.55±2.88	86.38±1.01
48	67.51±1.32	92.46±2.40
72	75.64±2.68	97.63±1.09
96	85.61±2.89	

Figure 5.25: Effect of presence of collagenase in the dissolution medium on the release of celecoxib from chitosan microspheres

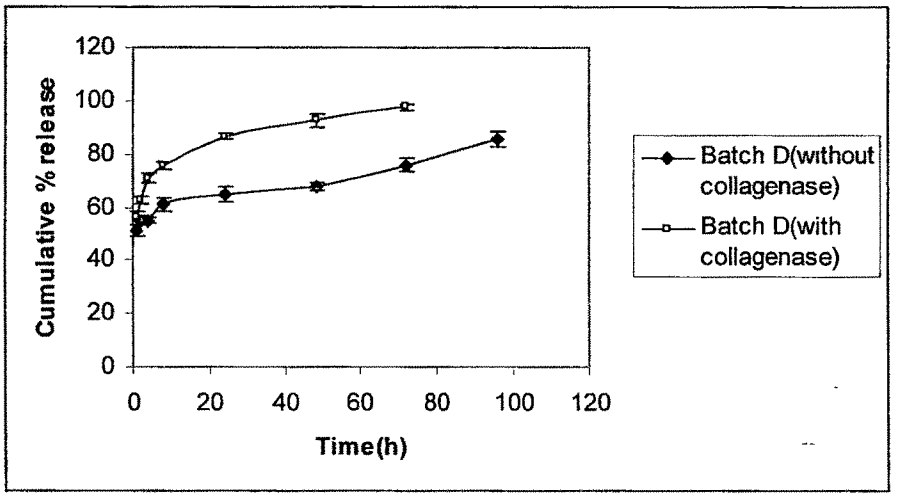


Table 5.50: In-vitro release profile of celecoxib loaded chitosan microspheres (fitted to Korsemeyer and Peppas model)

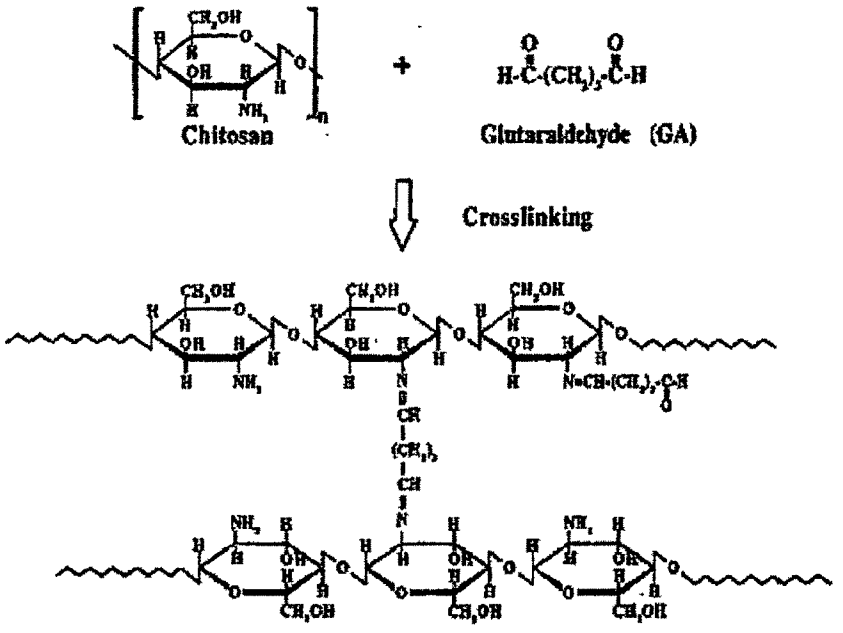
Log t	Log(Qt/Qα)											
	A	B	C	D	E	F	G	H	J	K	CMS-4	D-collagenase
0 000	-0 183	-0 237	-0.273	-0 295	-0 182	-0 218	-0 266	-0 269	-0 150	-0 133	-0 184	-0 253
0 301	-0.162	-0 223	-0 238	-0 282	-0 167	-0 184	-0 217	-0 230	-0 091	-0 065	-0 158	-0 205
0 602	-0 121	-0 112	-0.218	-0 262	-0 120	-0 162	-0 183	-0 207	-0 043	-0 034	-0 123	-0 151
0 903	-0.091	-0.093	-0 182	-0 217	-0 082	-0 154	-0 161	-0 182	-0 019	-0 016	-0 093	-0 121
1.380	-0 055	-0 078	-0 142	-0 190	-0 043	-0.094	-0 122	-0 132			-0 053	-0 064
1 681	-0 014	-0 073	-0 105	-0 171	-0 012	-0 051	-0 048	-0 075			-0 021	-0 034
1 857		-0 018	-0 080	-0 121		-0 019	-0 018	-0 043			-0 005	-0 010
1 982		-0 010	-0 019	-0 067								

Table 5.51: Release kinetic parameters of celecoxib loaded chitosan microspheres

Batch code	Correlation coefficient (r^2)				n (release exponent)	K (release rate constant)
	Zero order	Higuchi	First order	Peppas		
A	0.862	0.957	0 982	0.992	0.100	0.653
B	0.721	0.820	0.916	0.889	0.107	0.599
C	0.943	0.972	0.895	0.958	0.113	0.524
D	0.954	0.950	0.940	0.910	0.099	0.495
E	0.825	0.939	0.985	0.988	0.105	0.651
F	0.941	0.991	0.988	0.969	0.102	0.597
G	0.929	0.978	0.982	0.971	0.124	0.544
H	0.938	0.991	0.993	0 979	0.116	0.533
J	0.826	0.916	0.967	0.968	0.146	0.721
K	0.744	0.848	0.940	0.919	0.127	0.758
CMS-4	0.744	0.848	0.940	0.999	0.127	0.758
D-collagenase	0.815	0.932	0.980	0.990	0.126	0.574

As shown in figure 5.20, an increase in the concentration of glutaraldehyde led to a decrease in the rate of drug release. Microspheres prepared using 1.0 ml of glutaraldehyde (Batch C, Batch D) releases the drug slowly compared to that prepared with 0.5 ml of glutaraldehyde (Batch A, Batch B). However, a burst effect was observed in all the formulations. In general, around 50% of the drug is released in the first hour, followed by slower release for a period of 96 hours.

Similar results were obtained for formaldehyde cross-linked microspheres. Increasing the formaldehyde volume and duration of cross-linking leads to a decrease in the drug release rate. Microspheres prepared using 1.0 ml formaldehyde (Batch G, Batch H) releases the drug slowly compared to the microspheres prepared using 0.5 ml formaldehyde (Batch E, Batch F). However, a burst effect was observed in all the formaldehyde cross-linked microspheres. About 60% of the drug is released in the first hour followed by a controlled release for a period of 72 hours. There is a significant difference ($p<0.05$) in the drug release rates of the formaldehyde cross-linked microspheres and the glutaraldehyde cross-linked microspheres. The release rate of celecoxib from formaldehyde crosslinked microspheres is significantly higher than that of the glutaraldehyde crosslinked microspheres. Since glutaraldehyde possesses two aldehyde groups, one molecule of glutaraldehyde can react with two chitosan molecules as shown in the following scheme:



The duration of cross-linking also had an effect on the drug release. An increase in the duration of cross-linking from 1 hour to 3 hours led to a decrease in the drug release as shown in Figures 5.20 and 5.21 respectively for glutaraldehyde and formaldehyde cross-linked microspheres.

The heat cross-linked microspheres shows the fastest drug release. About 70% of the drug is released in the first hour and 95% of the drug is released in 8 hours. As shown in figure 5.23, the drug release rate from the heat cross-linked microspheres is significantly higher ($p<0.05$) than the chemically cross-linked microspheres. The results are in concurrence with the earlier reports (Kumbhar et al, 2002). This may be because of the chemical reaction of chitosan with formaldehyde or glutaraldehyde leading to a stronger and a more rigid matrix than the heat cross-linked microspheres. No significant difference was observed between the release rates of microspheres prepared at 70°C or 90°C. However a burst effect which may be attributed to the drug present on the surface of the microspheres is observed in all the formulations. The release kinetic parameters of

celecoxib loaded chitosan microspheres are shown in table 5.51. The value of the correlation coefficient (r^2 values) calculated by fitting the data to zero order, higuchi, first order kinetics and Korsemeyer and Peppas model models with respect to the optimized batch D is closer to 1 for zero order. This indicates that the release of the optimized batch D is best explained by zero order. The value of release exponent n for all the batches is less than 0.5 indicating that the mechanism of celecoxib release from the microspheres is quasi fickian diffusion. The value of release rate constant K decreases with an increase in the volume of formaldehyde or glutaraldehyde indicating that the release rate decreases with an increase in the cross-linking density. The K value of microspheres crosslinked using glutaraldehyde or formaldehyde is lower than that of the heat crosslinked microspheres indicating the higher release rate from heat crosslinked microspheres. The release studies conducted in presence of collagenase indicated that the release rate of celecoxib increases in presence of collagenase which may be probably due to the partial breakdown of the chitosan microspheres mediated by the enzyme collagenase.

The effect of the different variables on the release of rofecoxib from chitosan microspheres is shown in tables 5.52 to 5.53 and figures 5.26 and 5.27. The release kinetic parameters of rofecoxib loaded chitosan a microsphere is shown in table 5.55.

Table 5.52: Effect of volume of glutaraldehyde on the release of rofecoxib from chitosan microspheres

Time(h)	Cumulative % release	
	R-CM7 (0.1ml glutaraldehyde)	R-CM8 (0.25 ml glutaraldehyde)
1	50.51±1.46	55.41±2.46
2	53.84±1.12	61.67±1.40
3	59.61±2.01	65.25±1.40
4	65.38±1.22	69.05±2.19
8	74.96±1.20	77.48±1.95
24	84.92±1.68	84.69±1.33
48	97.38±1.06	95.73±0.54

Figure 5.26: Effect of volume of glutaraldehyde on the release of rofecoxib from chitosan microspheres

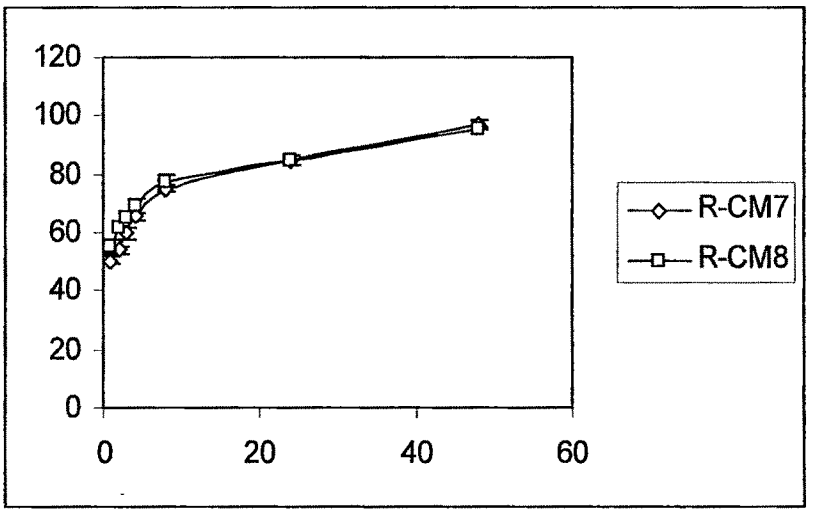


Table 5.53: Effect of chitosan concentration on the rofecoxib release from chitosan microspheres

Time(h)	Cumulative % release	
	R-CM1 (2%w/w chitosan)	R-CM7 (3% w/w chitosan)
1	52.37±1.77	50.51±1.46
2	73.11±0.95	53.84±1.12
3	87.5±2.81	59.61±2.01
4	90.27±2.58	65.38±1.22
8	97.32±1.15	74.96±1.20
24		84.92±1.68
48		97.38±1.06

Figure 5.27: Effect of chitosan concentration on release of rofecoxib from chitosan microspheres

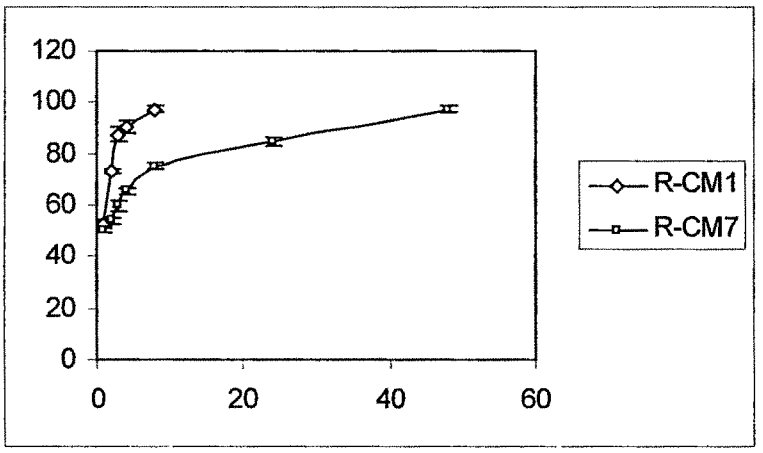


Table 5.54: In-vitro release profile of rofecoxib from chitosan microspheres (fitted to Korsemeyer-peppas model)

Log t	Log(Qt/Qα)		
	R-CM7	R-CM8	R-CM1
0.000	-0 297	-0.256	-0.281
0 301	-0.269	-0.210	-0.136
0.477	-0.225	-0.185	-0 058
0.602	-0 185	-0 161	-0 044
0.903	-0.125	-0.111	-0 012
1 380	-0.071	-0.072	
1.681	-0.012	-0 019	

Table 5.55: Release kinetic parameters of rofecoxib loaded chitosan microspheres

Batch code	r^2				n (peppas model)	K (peppas model)
	Zero order	Higuchi	First order	peppas		
R-CM7	0.853	0.949	0.980	0.983	0.173	0.498
R-CM8	0.845	0.943	0.975	0.989	0.137	0.562
R-CM1	0.677	0.809	0.958	0.884	0.300	0.571

n= Release exponent
K= Release rate constant

It is evident from figures 5.26 and 5.27 that the main factor affecting the release of rofecoxib from chitosan microspheres is the concentration of chitosan. The volume of glutaraldehyde has no significant influence on the drug release. The reason behind this may be the low volume of glutaraldehyde used for the preparation of rofecoxib loaded chitosan microspheres while at higher volume the microspheres are not formed. The low level used may not be sufficient to control the release of rofecoxib from the microspheres. The concentration of chitosan has a significant influence on the release of rofecoxib from the microspheres. The release rate of rofecoxib from the microspheres prepared using 2% chitosan (R-CM1) is significantly faster than those prepared using 3% chitosan (R-CM7). The values of r^2

obtained for the various models indicate that for batches R-CM7 and R-CM8, rofecoxib is released from the microspheres as per the korsemeyer and peppas model while batch R-CM1 releases rofecoxib as per first order kinetics. The value of the release exponent n is less than 0.5 indicating a quasi-fickian diffusion mechanism of drug release. The release rate constant of batch R-CM7 which is crosslinked using 0.1 ml glutaraldehyde is not significantly different from that of R-CM8 which is crosslinked using 0.25 ml glutaraldehyde. Hence it can be inferred that there is no significant effect of the volume of glutaraldehyde on the release rate of rofecoxib from chitosan microspheres.

The effect of the different variables on the release of valdecoxib from chitosan microspheres is shown in tables 5.56 and 5.57 and figures 5.28 and 2.29. The release kinetic parameters of the valdecoxib loaded chitosan microspheres are shown in table 5.59.

Table 5.56: Effect of volume of glutaraldehyde on release of valdecoxib from chitosan microspheres

Time(h)	Cumulative % release		
	V-CM6 (0.5 ml)	V-CM7 (1.0 ml)	V-CM8 (1.5 ml)
1	70.84±2.08	55.13±1.47	54.2±1.13
2	76.82±0.78	60.4±0.94	61.83±1.46
4	84.58±2.25	64.97±1.46	66.38±0.85
8	95.17±1.67	70.36±1.04	73.51±0.31
24		77.82±1.46	83.16±1.67
48		82.28±1.23	88.6±1.64
72		88.37±0.71	93.58±1.29
96		96.52±1.26	

Figure 5.28: Effect of volume of glutaraldehyde and duration of cross-linking on release of valdecoxib from chitosan microspheres

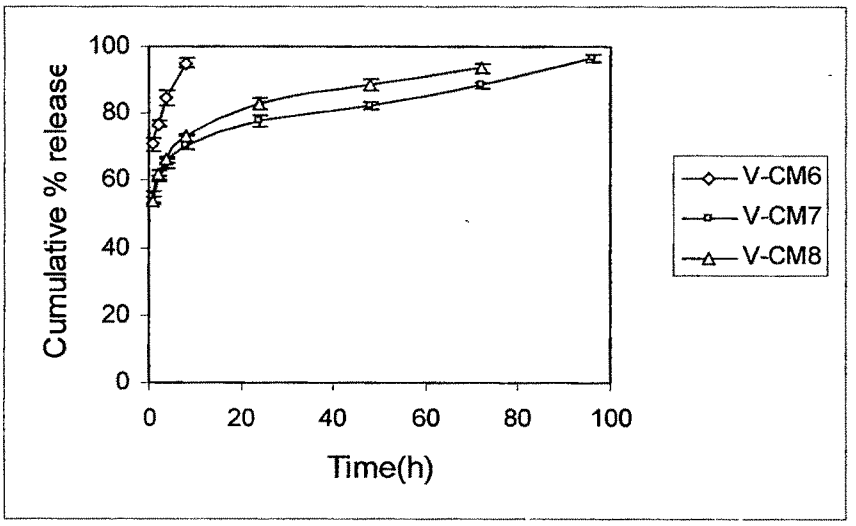


Table 5.57: Effect of chitosan concentration on release of valdecoxib from chitosan microspheres

Time(h)	Cumulative % release	
	V-CM2 (2% w/w)	V-CM7 (3%w/w)
1	60.73±2.38	55.13±1.47
2	67.84±3.01	60.4±0.94
4	73.2±1.58	64.97±1.46
8	79.56±1.48	70.36±1.04
24	88.58±1.56	77.82±1.46
48	95.77±1.26	82.28±1.23
72		88.37±0.71
96		96.52±1.26

Figure 5.29: Effect of chitosan concentration on release of valdecoxib from chitosan microspheres

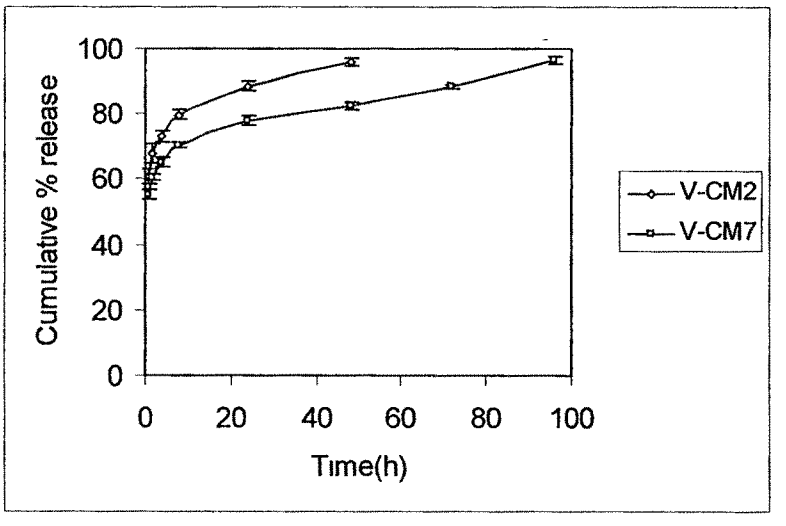


Table 5.58: In-vitro release profile of valdecoxib loaded chitosan microspheres (fitted to Korsemeyer-peppas model)

Log t	Log(Qt/Qα)			
	V-CM6	V-CM7	V-CM8	V-CM2
0 000	-0 150	-0 259	-0.266	-0.217
0.301	-0.115	-0.219	-0.209	-0.169
0.602	-0.073	-0.187	-0.178	-0.135
0.903	-0.021	-0 153	-0.134	-0 099
1.380		-0.109	-0.080	-0.053
1.681		-0.085	-0 053	-0.019
1 857		-0 054	-0.029	
1 982		-0.015		

Table 5.59: Release kinetic parameters of valdecoxib loaded chitosan microspheres

Batch code	r^2				n (peppas model)	K (peppas model)
	Zero order	Higuchi	First order	peppas		
V-CM6	0.975	0.999	0.994	0.993	0.142	0.701
V-CM7	0.901	0.969	0.935	0.985	0.112	0.554
V-CM8	0.818	0.935	0.962	0.992	0.123	0.558
V-CM-2	0.826	0.940	0.981	0.994	0.114	0.619

n= release exponent
K= Release rate constant

It is evident from the release studies of valdecoxib from chitosan microspheres that with an increase in the chitosan concentration, there is a decrease in the release of valdecoxib from the microspheres. As the volume of glutaraldehyde is increased from 0.5 ml (V-CM6) to 1.0 ml (V-CM7), there is a decrease in the release rate. With further increase in the glutaraldehyde volume to 1.5 ml (V-CM8) there is an increase in the release rate as evidenced from the increased value of the release rate constant. Hence, 1.0 ml glutaraldehyde can be considered as an optimum volume for crosslinking of microspheres. The release of

valdecoxib from chitosan microspheres from all the batches can be best explained by the Korsemeyer-peppas model (except batch V-CM6 which releases the drug as per Higuchi model) as indicated by the r^2 values shown in table 5.59. The value of the release exponent n in all the batches is less than 0.5 indicating that the release of valdecoxib from chitosan microsphere is by quasi-fickian diffusion.

5.10.3.3 Residual glutaraldehyde content

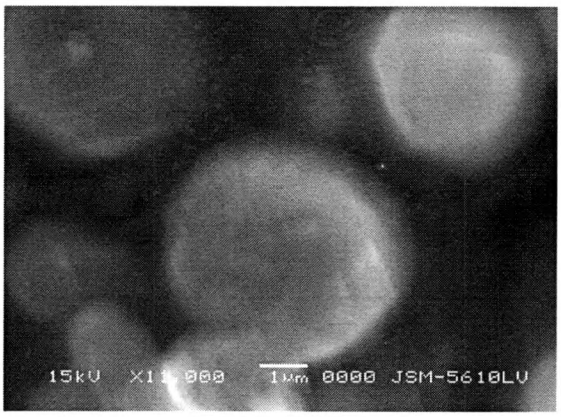
The residual glutaraldehyde and formaldehyde content determined in the microspheres was found to be less than 5 ppm.

5.10.3.4 Scanning electron microscopy

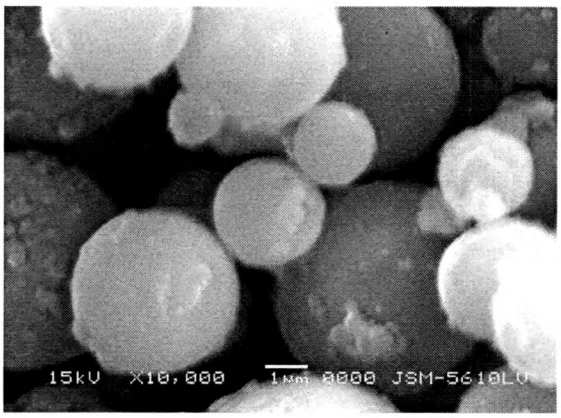
The scanning electron microscopy studies revealed that the surface of the microspheres is rough indicating the presence of celecoxib on the surface of the microspheres.

Figure 5.30: Scanning electron micrographs of celecoxib loaded chitosan microspheres

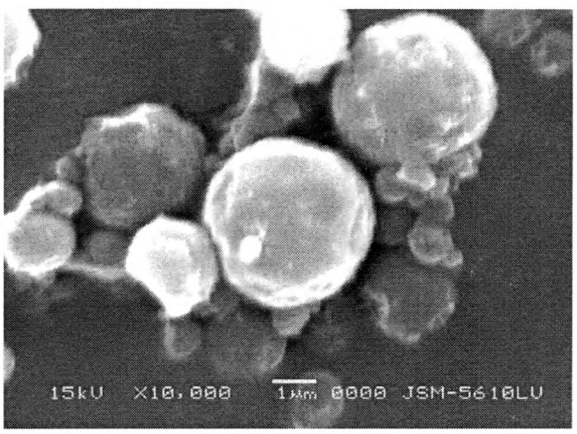
Glutaraldehyde crosslinked



Formaldehyde crosslinked



Heat crosslinked



5.10.3.5 FTIR Studies

Chitosan is a heteropolymer made up of glucosamine and acetylglucosamine units and its FTIR spectrum is characterized by several amine and amide bands in the region 1200-1800 cm^{-1}. The FTIR spectrum of chitosan is shown in figure 5.31. The amide-I band (conjugation of –NH deformation mode with C=O and C=N stretching modes) is present at 1651cm^{-1}. The amide–II band due to the N-H bending vibrations is present at 1558cm^{-1}. The presence of an absorption band at 3421cm^{-1} is due to the –NH stretching frequency of the amine group. The amide III band is present at 1338cm^{-1} which is due to the conjugation of –NH deformation mode with -C=O and -C=N stretching modes. The presence of a band at 2927 cm^{-1} is due to the –C-H stretching vibrations.

Figure 5.32 and 5.33 shows the FTIR spectrum of chitosan microspheres crosslinked with formaldehyde and glutaraldehyde respectively. In both the spectra, appearance of an extra weak band at around 1700cm^{-1} indicates the presence of the residual aldehyde used as cross-linking agent. The intensity of the band at 1700cm^{-1} being very weak, it was adjudged that the residual aldehyde content is very less and is within the limit as confirmed by the test for residual formaldehyde (IP) or glutaraldehyde (USP).

Figure 5.34 shows the FTIR spectrum of heat crosslinked chitosan microspheres.

The absence of the characteristic absorption bands of celecoxib in the FTIR spectra of celecoxib loaded chitosan microspheres indicates that celecoxib is entrapped in the microspheres and is not present in the free form in the microspheres.

The appearance of the new bands at 1562cm^{-1} in the FTIR spectrum of the formaldehyde and glutaraldehyde crosslinked chitosan microspheres is due to the

formation of imine group which shows a C=N stretch in the 1689-1471cm^{-1} region (Silverstein. 1991).

Figure 5.31: FTIR spectrum of chitosan

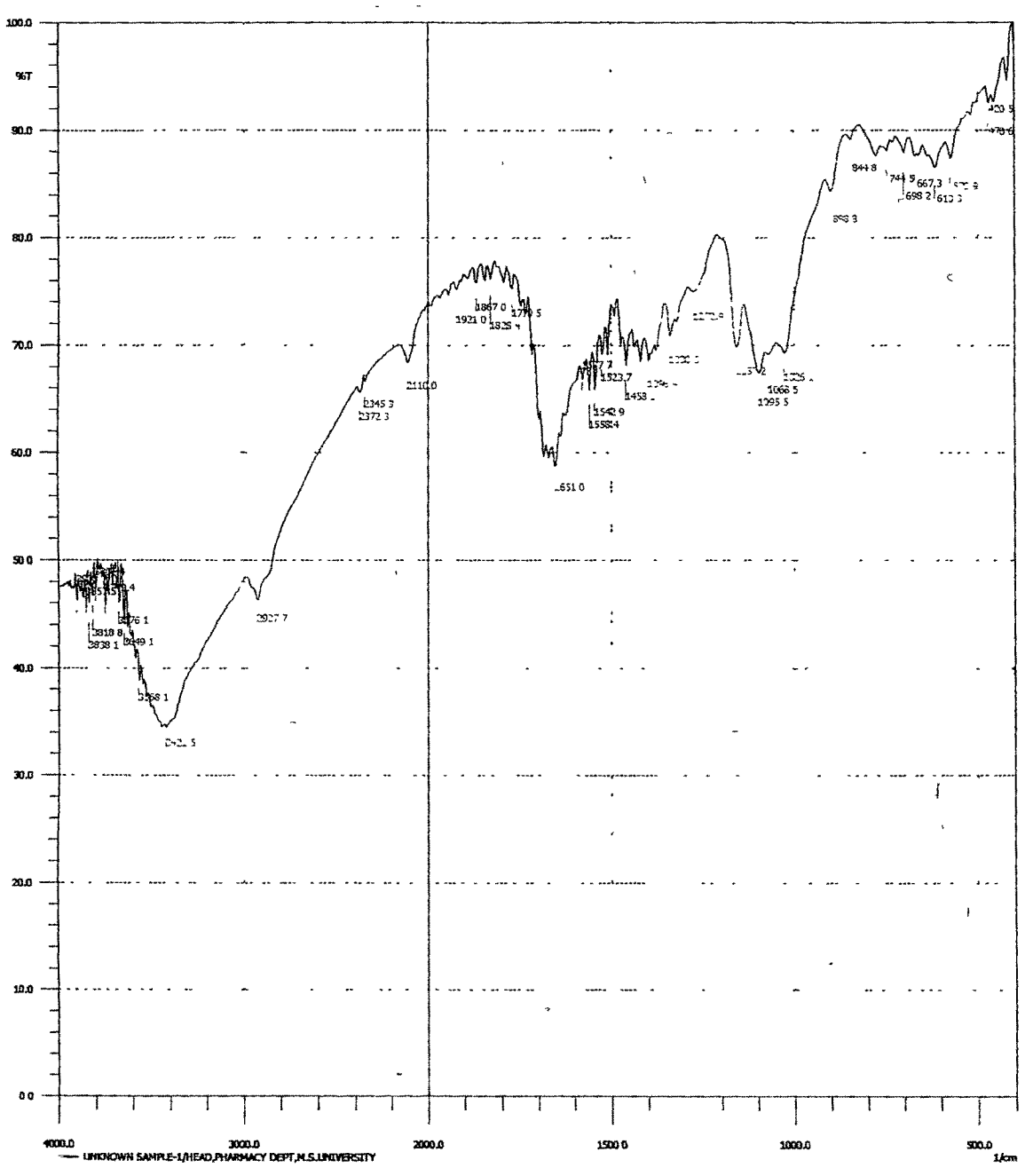


Figure 5.32: FTIR spectrum of formaldehyde crosslinked chitosan microspheres

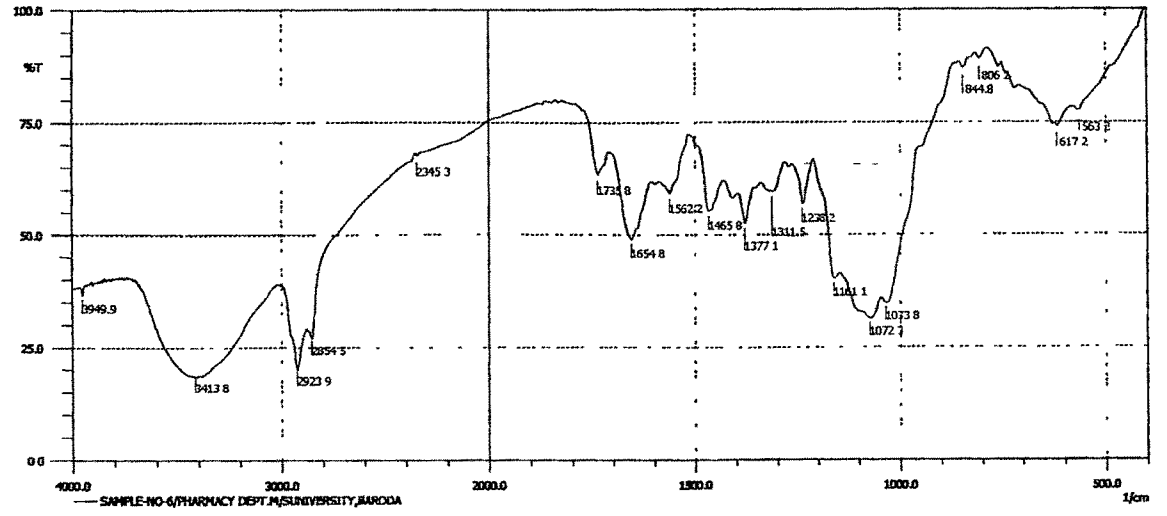


Figure 5.33: FTIR spectrum of glutaraldehyde crosslinked chitosan microspheres

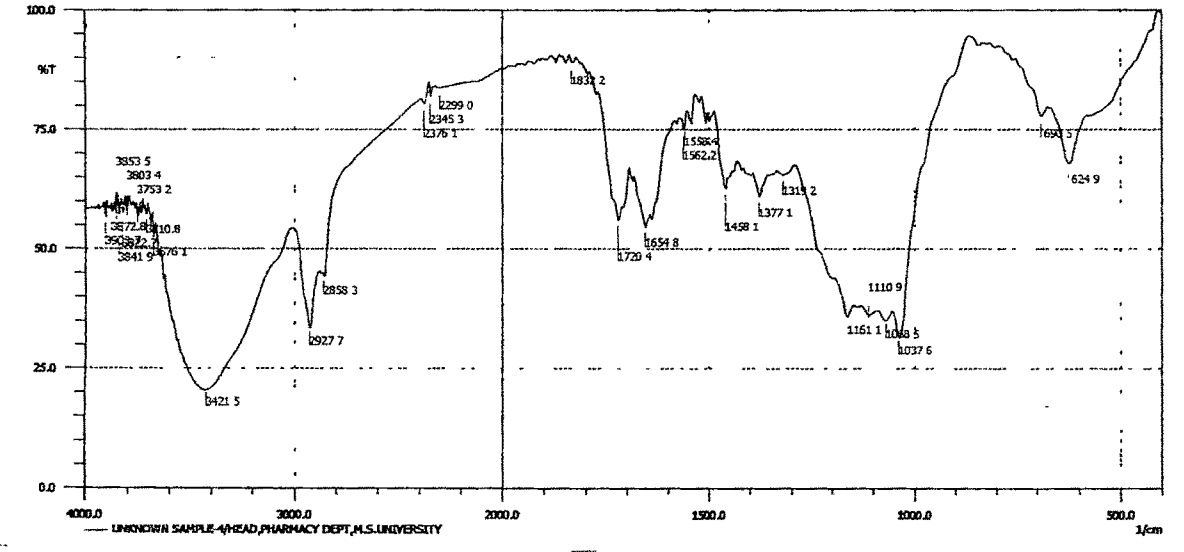
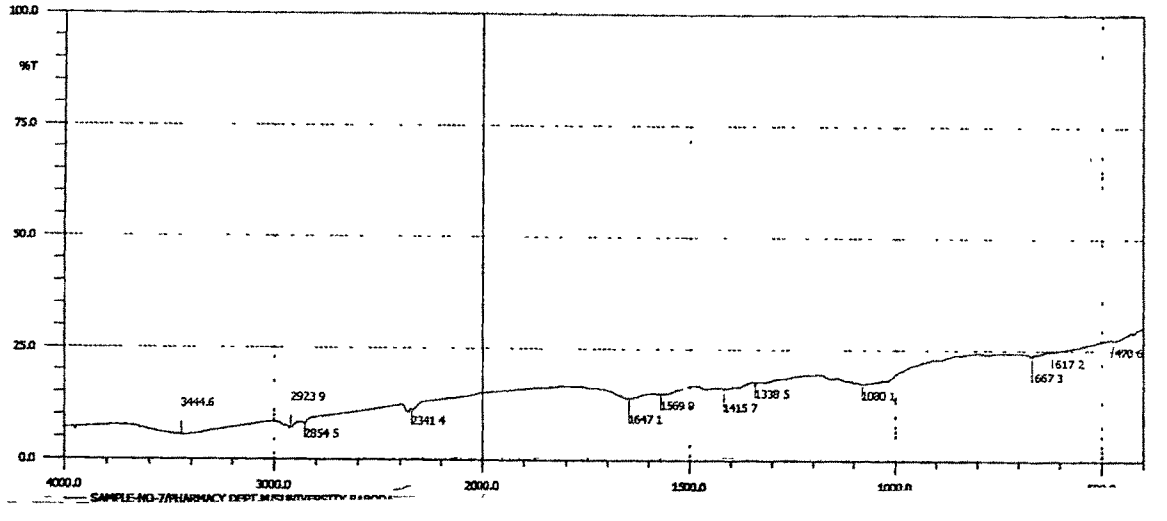


Figure 5.34: FTIR spectrum of heat crosslinked chitosan microspheres



5.10.4 Albumin microspheres

Various methods have been reported for the preparation of albumin microspheres. Thermal denaturation and chemical cross-linking using different cross-linking agents are the widely used methods for the preparation of albumin microspheres. The use of thermal denaturation has an advantage over the chemical cross-linking method due to the toxic nature of the chemical cross-linking agents glutaraldehyde and formaldehyde. However, thermal denaturation uses temperatures as high as 100°C -120°C which may degrade the thermolabile drugs. Hence it cannot be used for thermolabile drugs. Celecoxib is not a thermolabile drug. Hence thermal denaturation was used as one of the methods for the preparation of celecoxib loaded albumin microspheres. Many reports are available where microspheres are produced by chemical cross-linking with glutaraldehyde. However, only one report involves the use of formaldehyde as a cross-linking agent (Katti and Krishnamurti, 1999). An attempt has been made to compare the effect of the preparation method on the characteristics of bovine serum albumin microspheres.

5.10.4.1 Entrapment efficiency and particle size

The entrapment efficiency of the albumin microspheres was found out by degrading the microspheres in presence of pepsin. The preliminary experiments revealed that the albumin microspheres did not degrade completely by treatment with 0.1N HCl or NaOH. So the microspheres were treated with pepsin in 0.1N HCl. The microspheres released the content completely in presence of pepsin. The drug is then extracted with dichloromethane and the organic extract containing the drug is evaporated to

dryness. The residue is dissolved in methanol and the absorbance of the resulting solution is measured at the λmax of the drug. The entrapment efficiency is then found as per the formula given in section 5.4.

5.10.4.1a Effect of method of preparation

Two different methods viz. Thermal denaturation and emulsification chemical cross-linking were used to prepare celecoxib loaded bovine serum albumin microspheres. The microspheres prepared using thermal denaturation had significantly low entrapment efficiency than those prepared using emulsification chemical cross-linking method. The reason behind this is the solubility of celecoxib in the external phase of the w/o emulsion at high temperature (100°C) used for the preparation of the microspheres. The particle size of the microspheres prepared using thermal denaturation is also significantly less than that of the microspheres prepared using emulsification chemical cross-linking method. The high temperature used for the preparation of microspheres leads to complete evaporation of water from the internal phase and shrinkage of the microspheres. Hence low particle size is obtained for microspheres prepared using thermal denaturation. As shown in table 5.60, no microspheres were obtained at 70°C instead a slimy layer was obtained on the filter which could not be separated in the form of microspheres. This indicates heating at this temperature may not be sufficient to cause denaturation of albumin. Temperatures as high as 130°C have been used for denaturation of albumin by previous workers (Filipovic and Jalsenjak, 1993).

Albumin microspheres with high entrapment efficiency were prepared using the emulsification chemical cross-linking technique. The high entrapment efficiency of celecoxib observed here is due to the insolubility of celecoxib in the external phase and the cross-linking agents formaldehyde and glutaraldehyde under the conditions used for the preparation of microspheres. There was no significant difference in the entrapment efficiency between formaldehyde and glutaraldehyde crosslinked microspheres. Both the cross-linking agents gave microspheres having high entrapment efficiencies ranging from 85% to 95%.

Table 5.60: Effect of temperature on the entrapment efficiency and particle size of celecoxib loaded albumin microspheres prepared by thermal denaturation

Batch code	Concentration of span-85 %w/w	Temperature (°C)	% Entrapment efficiency	Particle size (μm)
TD-1	0	70	No microspheres were formed	
TD-2	2	70	No microspheres were formed	
TD-3	2	100	23.04±1.83	4.13±1.62
TD-4	5	100	20.55±2.87	3.82±1.16

Table 5.61: Entrapment efficiency and particle size of the celecoxib loaded albumin microspheres prepared by emulsification chemical cross-linking method

Batch code	Cross-linking agent	% Entrapment efficiency	Particle size (μm)
C-FA	Formaldehyde	88.74±2.08	9.53±1.50
C-GA	Glutaraldehyde	90.63±1.90	5.46±0.31

5.10.4.1b Effect of concentration of BSA, concentration of span-85 and volume of crosslinking agent

The entrapment efficiency was found to be dependent on the concentration of albumin and concentration of span-85. As shown in table 5.61 and 5.62, an increase in the concentration of albumin from 20%w/w to 30% w/w led to a significant increase ($p<0.05$) in the entrapment efficiency. There was a significant decrease in the entrapment efficiency with an increase in the concentration of span-85 from 2%w/w to 5%w/w and the decrease was more pronounced at lower albumin concentration. There was no significant difference in the entrapment efficiencies of microsphere crosslinked with formaldehyde and glutaraldehyde. The volume of the crosslinking agent had no significant influence on the entrapment efficiency of the microspheres as shown in tables 5.62 and 5.63.

The particle size of the microspheres was dependent on the concentration of bovine serum albumin, concentration of span-85 and the crosslinking agent used. There was an increase in the particle size with an increase in the bovine serum albumin concentration while a decrease in the particle size was observed with an increase in the span-85 concentration. The decrease in the particle size with an increase in the emulsifier concentration was more pronounced at lower concentration of albumin. The microspheres crosslinked using formaldehyde had significantly higher particle size than those crosslinked using glutaraldehyde. The volume of formaldehyde or glutaraldehyde had no significant influence on the particle size of the microspheres as can be seen from tables 5.62 and 5.63.

Table 5.62: Effect of albumin concentration, span-85 concentration and volume of formaldehyde on the particle size and entrapment efficiency of celecoxib loaded albumin microspheres

Batch code	Concentration of BSA (%w/w)	Concentration of Span-85 (%w/w)	Volume of formaldehyde (ml)	Entrapment efficiency (%)	Particle size (μm)
C-FA1	20	2.0	0.5	72.72±1.98	7.64±0.78
C-FA2	20	2.0	1.0	71.80±1.37	6.89±0.37
C-FA3	20	5.0	0.5	60.30±0.84	5.32±1.78
C-FA4	20	5.0	1.0	58.48±2.39	5.41±0.24
C-FA5	30	2.0	0.5	90.27±1.02	11.74±0.81
C-FA6	30	2.0	1.0	92.30±2.87	11.02±1.88
C-FA7	30	5.0	0.5	85.62±1.05	9.53±0.61
C-FA8	30	5.0	1.0	87.71±0.70	8.62±1.28

Table 5.63: Effect of albumin concentration, span-85 concentration and volume of glutaraldehyde on the particle size and entrapment efficiency of celecoxib loaded albumin microspheres

Batch code	Concentration of BSA (%w/w)	Span-85 concentration (%w/w)	Volume of Glutaraldehyde (ml)	% Entrapment efficiency	Particle size (μm)
C-GA1	20	2.0	0.5	70.52±1.40	4.42±0.28
C-GA2	20	2.0	1.0	68.31±1.77	4.11±0.13
C-GA3	20	5.0	0.5	55.24±1.68	3.22±0.19
C-GA4	20	5.0	1.0	57.19±0.88	3.05±0.12
C-GA5	30	2.0	0.5	88.58±0.95	6.29±0.52
C-GA6	30	2.0	1.0	90.63±1.90	5.46±0.31
C-GA7	30	5.0	0.5	82.64±3.66	3.71±0.08
C-GA8	30	5.0	1.0	80.51±2.72	4.13±0.10
C-GA9	30	2.0	1.5	88.61±1.73	5.03±0.23

5.10.4.1c Effect of stirring speed

As shown in table 5.64, as the stirring speed is increased from 1500 rpm to 2500 rpm, there was no significant effect on the entrapment efficiency but the particle size decreased from 8.80μm to 5.46μm. As the stirring speed was further increased from 2500 rpm to 4000 rpm, there was no significant reduction in the particle size. However, there was a reduction in the entrapment efficiency. This may be because at higher stirring speed, the dissolution of celecoxib in the external phase increases leading to decreased entrapment in the microspheres. Thus, owing to higher entrapment efficiency and lower particle size obtained at 2500 rpm, it was selected as an optimum stirring speed for the preparation of microspheres.

Table 5.64: Effect of stirring speed on the entrapment efficiency and particle size of the celecoxib loaded albumin microspheres

Stirring speed	% Entrapment efficiency	Particle size(μm)
1500	92.41±2.12	8.80±1.36
2500	90.63±1.90	5.46±0.31
4000	85.68±2.56	5.38±1.49

Albumin concentration: 3%w/w
Span-85 concentration: 2%w/w
Volume of glutaraldehyde: 1.0ml

5.10.4.1d Effect of water: oil phase volume ratio

Three different ratios of volume of water: oil phase viz. 1:10, 1:20 and 1:40 were used to find an optimum ratio. At the ratios of 1:10 and 1:20, the microspheres had a tendency to aggregate while; discrete microspheres were obtained at the ratio of 1:40. Thus, it was selected as an optimum phase volume ratio. No significant difference was observed between the entrapment efficiency of the batches prepared using different ratios. The

particle size of the batches prepared using 1:10 and 1:20 phase volume ratio was significantly higher than that obtained using 1:40 ratio of water: oil phase.

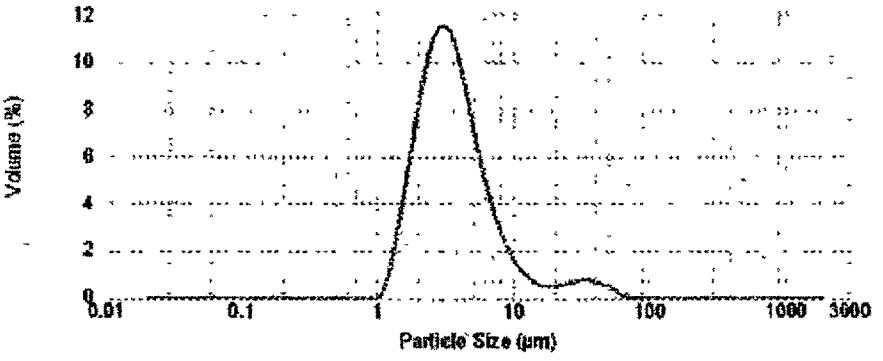
5.10.4.1e Effect of composition of external phase

Three different compositions of external phase were used to select an optimum composition. Light liquid paraffin, heavy liquid paraffin and a mixture of light: heavy liquid paraffin (1:1) was used to prepare the microspheres. It was observed that the microspheres prepared using light liquid paraffin were sticky in nature and had a tendency to aggregate. The reason may be the low viscosity of the light liquid paraffin which could not act as an efficient barrier to avoid agglomeration of the microspheres. However, a mixture of light liquid paraffin and heavy liquid paraffin (1:1) and heavy liquid paraffin gave discrete microspheres in the form of fine powder. The microspheres prepared using a mixture of light and heavy liquid paraffin were polydisperse having a wide size range of particles while those prepared using heavy liquid paraffin were monodisperse as shown in figure 5.35. Thus heavy liquid paraffin was selected as an ideal external phase for the preparation of microspheres. There was no significant effect of the composition of the external phase on the entrapment efficiency as can be seen from table 5.65.

Table 5.65: Effect of composition of external phase on the entrapment efficiency and the particle size of celecoxib loaded albumin microspheres

External phase	% Entrapment efficiency	Particle size (μm)
Light liquid paraffin	91.27±0.91	8.40±1.10
Heavy liquid paraffin	90.63±1.90	5.46±0.31
Light: Heavy liquid paraffin(1:1)	92.38±2.94	6.28±1.34

Figure 5.35: Particle size distribution of celecoxib loaded bovine serum albumin Microspheres



The data obtained from celecoxib loaded albumin microspheres shows that the entrapment efficiency of the microspheres prepared using thermal denaturation is very less and hence it is not a suitable method for the preparation of celecoxib loaded albumin microspheres. Out of the two crosslinking agents used viz, formaldehyde and glutaraldehyde, the glutaraldehyde crosslinked microspheres were found to have lower particle size than the formaldehyde crosslinked microspheres and hence glutaraldehyde is more promising than formaldehyde as a crosslinking agent. Thus studies on the rofecoxib and valdecoxib loaded albumin microspheres was done using glutaraldehyde as a cross-linking agent.

The effect of the concentration of albumin, span-85 and volume of glutaraldehyde on the entrapment efficiency of rofecoxib and valdecoxib loaded albumin microspheres is shown in tables 5.66 and 5.67 respectively.

Table 5.66: Effect of concentration of bovine serum albumin, span-85 and volume of glutaraldehyde on the entrapment efficiency and particle size of rofecoxib in albumin microspheres

Batch code	Concentration of BSA (%w/w)	Concentration of span-85 (%w/w)	Volume of glutaraldehyde (ml)	% Entrapment efficiency	Particle size (μm)
R-AMS1	20	2	0.5	67.94±2.13	6.38±0.38
R-AMS2	20	2	1.0	66.02±2.34	5.70±0.16
R-AMS3	20	2	1.5	65.83±1.27	5.83±0.30
R-AMS4	20	5	0.5	65.27±2.07	4.33±0.85
R-AMS5	20	5	1.0	67.04±1.60	4.01±1.17
R-AMS6	20	5	1.5	70.51±1.71	4.74±0.18
R-AMS7	30	2	0.5	92.63±1.83	9.20±0.45
R-AMS8	30	2	1.0	92.84±2.21	8.38±1.01
R-AMS9	30	2	1.5	89.18±1.67	8.05±1.08
R-AMS10	30	5	0.5	90.88±1.39	6.47±0.98
R-AMS11	30	5	1.0	89.17±1.82	6.55±0.58
R-AMS12	30	5	1.5	90.30±1.02	6.93±0.71

Table 5.67: Effect of concentration of bovine serum albumin, span-85 and volume of glutaraldehyde on the entrapment efficiency and particle size of rofecoxib in albumin microspheres

Batch code	Concentration of BSA (%w/w)	Concentration of span-85 (%w/w)	Volume of glutaraldehyde (ml)	% Entrapment efficiency	Particle size (μm)
V-AMS1	20	2	0.5	60.55±2.37	7.49±0.48
V-AMS2	20	2	1.0	58.04±1.91	7.06±0.60
V-AMS3	20	2	1.5	59.38±2.38	6.38±0.68
V-AMS4	20	5	0.5	53.40±2.85	4.17±0.53
V-AMS5	20	5	1.0	52.18±2.61	4.58±0.45
V-AMS6	20	5	1.5	55.74±2.00	5.30±0.28
V-AMS7	30	2	0.5	85.97±2.33	13.64±1.97
V-AMS8	30	2	1.0	84.61±1.96	11.58±2.68
V-AMS9	30	2	1.5	86.10±3.63	13.03±2.58
V-AMS10	30	5	0.5	80.37±2.93	8.53±0.92
V-AMS11	30	5	1.0	81.75±1.55	8.47±0.23
V-AMS12	30	5	1.5	79.43±3.39	7.88±0.64

Table 5.66 shows that the main factor affecting the entrapment of rofecoxib in albumin microspheres is the concentration of albumin. There is no significant effect of the concentration of span-85 and volume of glutaraldehyde on the entrapment efficiency of the microspheres. Unlike celecoxib, rofecoxib is not soluble in the external phase of the emulsion at the concentration of the span-85 used. Hence an increase in the span-85 concentration did not decrease the entrapment efficiency significantly.

The main factors affecting the particle size of the microspheres is the concentration of albumin and concentration of span-85. There was an increase in the particle size with an increase in the concentration of span-85 while a decrease in the particle size was observed with an increase in the

span-85 concentration. The volume of glutaraldehyde had no significant influence on the entrapment efficiency as well as particle size of the microspheres.

In case of valdecoxib loaded albumin microspheres, concentration of albumin and span-85 showed a significant influence on the entrapment efficiency and particle size of the microspheres. There was no significant influence of the volume of glutaraldehyde on the entrapment efficiency and particle size of the microspheres. With an increase in the concentration of albumin, there was an increase in the entrapment efficiency as well as particle size whereas a decrease in the entrapment efficiency as well as particle size was observed with an increase in the span-85 concentration. The batches V-AMS7 to V-AMS12 which were formulated using 30% albumin had high entrapment efficiencies of around 80%-85%. The batches V-AMS10 to V-AMS12 had entrapment efficiencies of around 80% and a low particle size of around 7μm-9μm. Hence these batches were studied for in-vitro drug release.

5.10.4.2 Drug release

The in-vitro release of celecoxib from albumin microspheres prepared using different methods is shown in tables 5.68 to 5.71 and figures 5.36 to 5.39. The release kinetic parameters of the celecoxib loaded albumin microspheres is shown in table 5.73.

Table 5.68: Effect of volume of formaldehyde on the release of celecoxib from albumin microspheres

Time(h)	Cumulative % release	
	C-FA7 (0.5 ml)	C-FA8 (1.0 ml)
1	61.84±2.42	55.16±1.53
2	69.40±2.51	62.71±1.97
4	77.38±2.64	70.42±2.99
8	85.17±1.66	81.39±3.72
24	96.25±1.47	87.64±1.98
48		95.18±1.66

Figure 5.36: Effect of volume of formaldehyde on the release of celecoxib from albumin microspheres

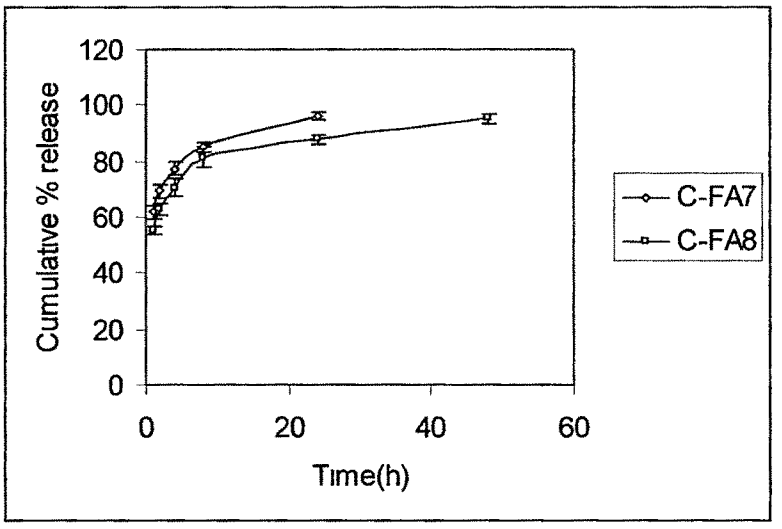


Table 5.69: Effect of volume of glutaraldehyde on the release of celecoxib from albumin microspheres

Time(h)	Cumulative % release		
	C-GA5 (0.5 ml)	C-GA6 (1.0 ml)	C-GA9 (1.5 ml)
1	53.4±1.10	41.33±1.04	19.56±0.51
2	58.99±1.22	48.73±1.14	20.09±0.66
4	61.45±0.62	49.51±0.87	22.93±0.21
8	64.36±1.49	55.64±1.55	27.01±0.81
24	66.51±0.94	60.88±0.45	28.13±0.73
48	70.91±0.35	63.24±1.76	34.62±1.59
72	78.49±1.08	70.36±1.33	41.02±1.18
96	85.82±0.43	75.81±1.86	47.38±1.47
144	91.56±1.29	85.64±1.45	52.41±1.30

Figure 5.37: Effect of volume of glutaraldehyde on the release of celecoxib from albumin microspheres

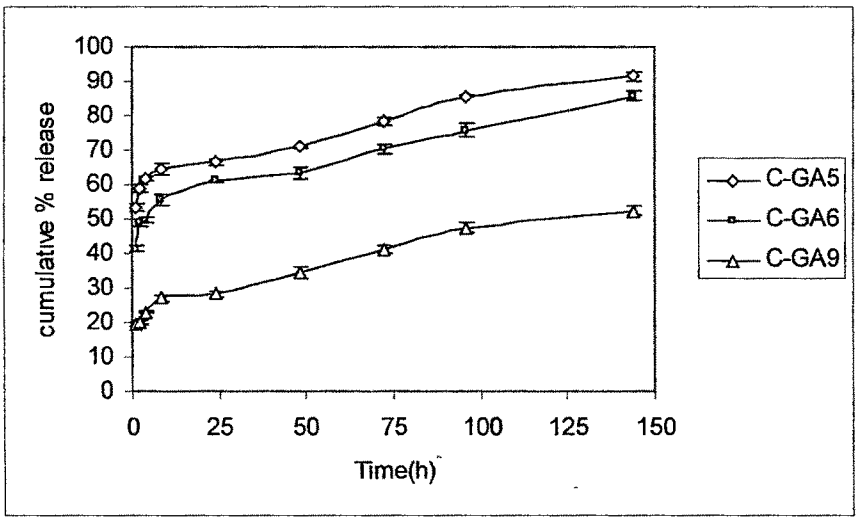


Table 5.70: In-vitro release profile of celecoxib loaded albumin microspheres prepared by thermal denaturation

Time(h)	Cumulative % release	
	TD-3 (2%w/w span-85)	TD-4 (5%w/w span-85)
1	80.41±2.56	82.17±1.81
2	87.29±2.68	89.05±3.42
4	95.63±1.08	97.64±1.16

Figure 5.38: In-vitro release profile of celecoxib loaded albumin microspheres prepared by thermal denaturation

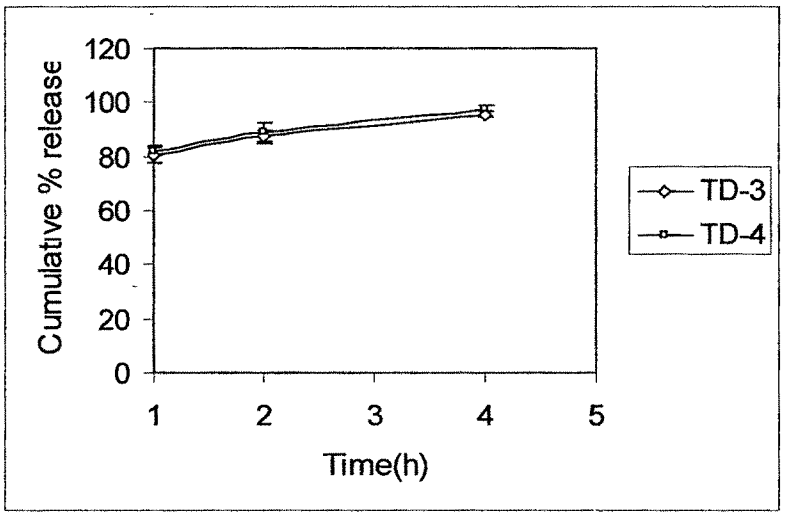


Table 5.71: Effect of presence of collagenase in the dissolution medium on the release of celecoxib from albumin microspheres

Time(h)	Cumulative % release of celecoxib from albumin microspheres(Batch C-GA6)	
	Without collagenase	With collagenase
1	41.33±1.04	48.95±1.79
2	48.73±1.14	55.26±2.36
4	49.51±0.87	64.97±2.39
8	55.64±1.55	70.4±2.64
24	60.88±0.45	78.52±1.99
48	63.24±1.76	86.15±1.19
72	70.36±1.33	94.73±2.32
96	75.81±1.86	
144	85.64±1.45	

Figure 5.39: Effect of presence of collagenase in the dissolution medium on the release of celecoxib from albumin microspheres

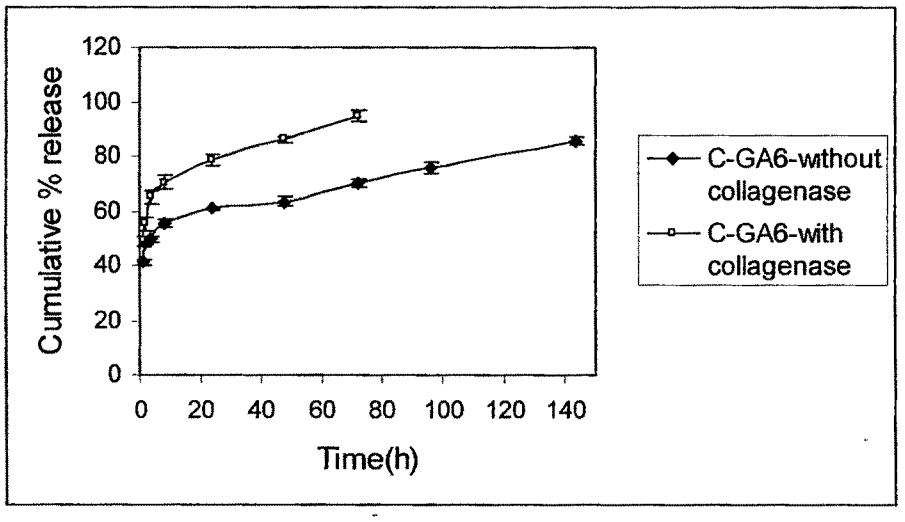


Table 5.72: In-vitro release profile of celecoxib from bovine serum albumin microspheres (fitted to Korsemeyer-peppas model)

Log t	Log(Qt/Qα)							
	C-FA7	C-FA8	TD-3	TD-4	C-GA5	C-GA6	C-GA9	C-GA6-collagenase
0.000	-0.209	-0.258	-0 095	-0.085	-0.272	-0.384	-0.709	-0.310
0.301	-0.159	-0.203	-0.059	-0.050	-0.229	-0.312	-0.697	-0.258
0.602	-0 111	-0.152	-0.019	-0.010	-0.211	-0.305	-0.640	-0.187
0 903	-0.070	-0 089			-0.191	-0.255	-0 568	-0.152
1.380	-0.017	-0.057			-0.177	-0.216	-0 551	-0.105
1.681		-0.021			-0.149	-0.199	-0.461	-0.065
1.857					-0.105	-0.153	-0.387	-0 024
1 982					-0.066	-0.120	-0 324	
2.158					-0.038	-0.067	-0.281	

Table 5.73: Release kinetic parameters of celecoxib loaded albumin microspheres

Batch code	r^2				n (Peppas model)	K (peppas model)
	Zero order	Higuchi	First order	Peppas		
C-FA7	0.821	0.935	0.986	0.991	0.139	0.629
C-FA8	0.750	0.885	0.946	0.969	0.139	0.571
TD-3	0.981	0.998	0.997	0.999	0.125	0.803
TD-4	0.984	0.998	0.989	0.998	0.124	0.820
C-GA5	0.945	0.965	0.978	0.915	0.094	0.532
C-GA6	0.923	0.968	0.980	0.955	0.126	0.431
C-GA9	0.958	0.970	0.977	0.940	0.196	0.177
C-GA6-collagenase	0.843	0.939	0.965	0.983	0.144	0.505

The results of the in-vitro release studies of celecoxib loaded albumin microspheres shows that the fastest release was observed in microspheres prepared by thermal denaturation while slowest release was observed in glutaraldehyde crosslinked microspheres. The formaldehyde crosslinked microspheres shows a release rate slower than that of microspheres prepared using thermal denaturation while faster than glutaraldehyde crosslinked microspheres. This can also be seen from table 5.73 which shows that the release rate constant of microspheres prepared by thermal denaturation is highest (K=0.820). The reason behind this may be the very small particle size of the microspheres. The diffusional path length that the drug has to traverse is reduced if the particle size of the microspheres is low. In addition to this, in case of chemical crosslinking, there is chemical reaction between albumin and crosslinking agent leading to stronger and more rigid matrices than obtained by thermal denaturation.

In order to evaluate the effect of volume of cross-linking agent on the release characteristics of the drug from the microspheres, formaldehyde was used in two different concentrations(0.5ml and 1.0ml) and glutaraldehyde was used in three different concentrations (0.5 ml, 1.0 ml and 1.5 ml). It was found that there was a decrease in the drug release with an increase in the volume of cross-linking agent as shown in figure 5.36 and 5.37. This is because of the formation of denser polymer cross-links and leading to increase in diffusional path length that the drug molecules have to traverse. This is in accordance with the earlier reports (Sahin et al, 2002). The release rate of celecoxib from albumin microspheres crosslinked using 1.5 ml glutaraldehyde is too low indicated by the very low value of the release rate constant (K=0.177). Hence further studies were done using batch C-GA6 which is prepared using 1.0 ml glutaraldehyde. The release profile of the batch C-GA6 was conducted

by adding collagenase in the release medium. The results indicated that there is a significant increase ($p<0.05$) in the release rate in presence of collagenase.

The kinetics of release of celecoxib from BSA microspheres showed a biphasic drug release pattern with an initial burst release followed by a slower release. This burst effect can be attributed to the presence of drug crystals on the surface of the microspheres. The data obtained from the drug release studies was subjected to model fitting and it was found that after the initial burst release in the first hour, the release of celecoxib from the microspheres can be best explained by the Korsemeyer and Peppas model for the formaldehyde crosslinked (Batch C-FA7 and C-FA8) and heat denatured microspheres(TD-3 and TD-4). The glutaraldehyde crosslinked microspheres (Batches C-GA5, C-GA6 and C-GA9) showed first order release as evidenced by the r^2 values. The value of n (release exponent) is less than 0.5 in all the batches indicating that celecoxib is released from the microspheres by quasi fickian diffusion.

The in-vitro release studies of celecoxib loaded albumin microspheres indicated that chemical crosslinked microspheres gave a controlled release while the heat crosslinked microspheres released the drug very rapidly. Out of the two crosslinking agents studied: formaldehyde and glutaraldehyde, glutaraldehyde seems to be more promising because of the slower release rates obtained. Thus albumin microspheres loaded with rofecoxib and valdecoxib were prepared using glutaraldehyde as a crosslinking agent.

The effect of glutaraldehyde volume on the release of rofecoxib from albumin microspheres is shown in table 5.74 and figure 5.40. The release kinetic parameters of rofecoxib loaded albumin microspheres is shown in table 5.76.

Table 5.74: Effect of volume of glutaraldehyde on the release of rofecoxib from albumin microspheres

Time(h)	Cumulative % release		
	R-AMS-10 (0.5 ml)	R-AMS-11 (1.0 ml)	R-AMS-12 (1 5 ml)
1	56.64±1.96	50.84±2.30	52.25±2.06
2	72.29±1.72	61.24±2.48	62.31±1 74
4	85 37±1.35	63 99±1.08	67.05±3.93
8	88.06±2.55	67.77±2.15	74.15±1.75
24	95.82±2.50	73.17±2.19	75.54±3.13
48		84.61±1.84	87.28±1.43
72		95.83±2.27	94.63±1.28

Figure 5.40: Effect of volume of glutaraldehyde on the release of rofecoxib From albumin microspheres

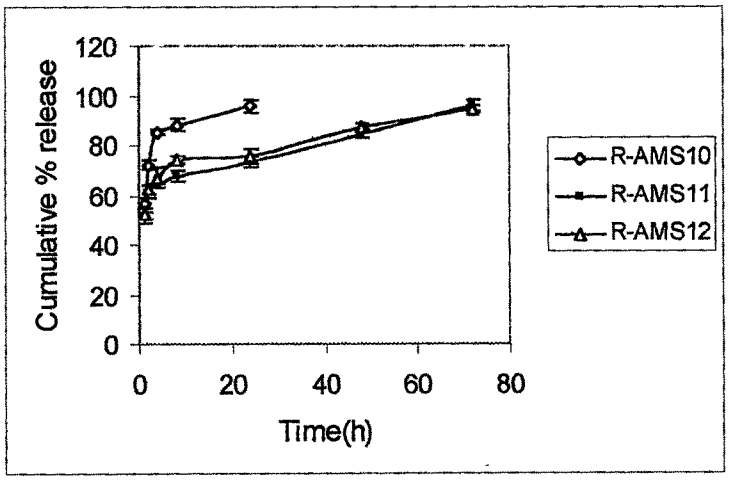


Table 5.75: In-vitro release of rofecoxib from albumin microspheres (fitted to peppas model)

Log t	Log(Qt/Qα)		
	R-AMS10	R-AMS11	R-AMS12
0.000	-0.247	-0.294	-0.282
0.301	-0.141	-0.213	-0.205
0.602	-0.069	-0.194	-0.174
0.903	-0.055	-0.169	-0.130
1.380	-0.019	-0.136	-0.122
1.681		-0.073	-0.059
1.857		-0.018	-0.024

Table 5.76: Release kinetic parameters of rofecoxib loaded albumin microspheres

Batch code	r^2				n (peppas model)	K (peppas model)
	Zero order	Higuchi	First order	peppas		
R-AMS10	0.575	0.730	0.860	0.855	0.156	0.622
R-AMS11	0.920	0.954	0.939	0.945	0.126	0.527
R-AMS12	0.845	0.918	0.957	0.948	0.121	0.552

The in-vitro release studies of rofecoxib loaded albumin microspheres shows that with an increase in the volume of glutaraldehyde from 0.5 ml (R-AMS10) to 1.0 ml(R-AMS11), there was a significant decrease in the drug release. With further increase in the volume of glutaraldehyde to 1.5 ml (R-AMS12), the release rate was not decreased significantly. Hence, 1.0 ml glutaraldehyde was considered as an optimum volume of glutaraldehyde for the preparation of microspheres. The release of valdecoxib from albumin microspheres from the optimized batch R-AMS11 can be best explained by the Higuchi model. The value of n (release exponent) in all the cases is less than 0.5 indicating a quasi fickian diffusion mechanism of the drug

release. The value of release rate constant K decreases with an increase in the glutaraldehyde volume from 0.5 ml to 1.0 ml. With further increase in the glutaraldehyde volume there is an increase in the release rate constant indicating an increase in the release rate. The reason behind this may be the formation of fractures on the surface of the microspheres with higher volume of glutaraldehyde.

Table 5.77 and shows the effect of glutaraldehyde volume on the release of valdecoxib from albumin microspheres. Table 5.79 depicts the release kinetic parameters of valdecoxib loaded albumin microspheres.

Table 5.77: Effect of volume of glutaraldehyde on release of valdecoxib from albumin microspheres

Time(h)	Cumulative % release		
	V-AMS10 (0.5 ml)	V-AMS11 (1.0 ml)	V-AMS12 (1.5 ml)
1	58.12±1.47	43.59±0.98	46.7±1.28
2	60.35±2.11	54.03±1.57	49.46±0.35
4	66.46±1.84	58.79±1.59	51.47±0.80
8	69.52±0.80	62.71±0.39	52.47±0.11
24	72.58±1.83	66.85±3.04	53.55±1.00
48	76.54±2.08	74.47±1.78	56.28±1.35
72	85.1±1.41	77.03±1.82	70.06±0.31
96	96.37±0.96	83.52±0.95	73.84±1.58
120		93.48±2.04	85.38±3.04
144			95.54±1.20

Figure 5.41: Effect of volume of glutaraldehyde on the release of valdecoxib from albumin microspheres

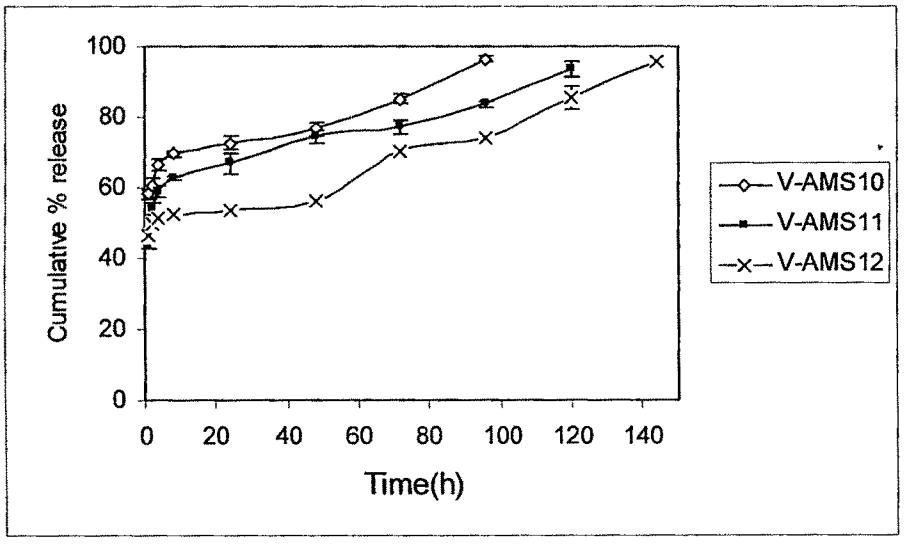


Table 5.78: In-vitro release of valdecoxib from albumin microspheres (fitted to Korsemeyer-peppas model)

Log t	Log (Qt/Qα)		
	V-AMS10	V-AMS11	V-AMS12
0.000	-0.236	-0.361	-0.331
0.301	-0.219	-0.267	-0.306
0.602	-0.177	-0.231	-0.288
0.903	-0.158	-0.203	-0.280
1.380	-0.139	-0.175	-0.271
1.681	-0.116	-0.128	-0.250
1.857	-0.070	-0.113	-0.155
1.982	-0.016	-0.078	-0.132
2.079		-0.029	-0.069
2.158			-0.020

Table 5.79: Release kinetic parameters of valdecoxib loaded albumin microspheres

Batch code	R^2				n (peppas model)	K (peppas model)
	Zero order	Higuchi	First	peppas		
V-AMS10	0.939	0.937	0.859	0.909	0.094	0.570
V-AMS11	0.889	0.938	0.910	0.946	0.129	0.467
V-AMS12	0.969	0.884	0.828	0.765	0.120	0.431

n= release exponent
K= release rate constant

The release studies of valdecoxib loaded albumin microspheres, it can be seen that with an increase in the glutaraldehyde volume from 0.5 to 1.5 ml of glutaraldehyde, there was a decrease in the drug release and hence 1.5 ml glutaraldehyde was considered as an optimum volume for the preparation of valdecoxib loaded albumin microspheres. The release rate constant decreases from 0.570 to 0.431 with an increase in the volume of glutaraldehyde from 0.1 ml to 1.5 ml indicating a decrease in the release

rate. The release of valdecoxib from albumin microspheres is best explained by zero order (except batch V-AMS11 which releases the drug as per the korsemeyer peppas model) as evidenced by the r^2 values. The value of the release exponent n is less than 0.5 in all the batches indicating that valdecoxib is released from the albumin microspheres by quasi fickian diffusion.

5.10.4.3 Residual cross-linking agent

The test for residual glutaraldehyde or formaldehyde conducted done on the microspheres indicates that the level of the residual cross-linking agent is less than 5 ppm. Thus, the microspheres are devoid of toxicity of glutaraldehyde or formaldehyde.

5.10.4.4 Scanning electron microscopy studies

The scanning electron micrographs as shown in figure 5.43 indicate that the surface of the microspheres is rough which may be due to the presence of the drug crystals on the surface. This is also reflected in the in-vitro drug release studies which shows burst effect owing to release of the surface associated drug. The surface of the plain microspheres is smooth as can be seen from figure 5.42.

Figure 5.42: Scanning electron micrograph of plain albumin microspheres

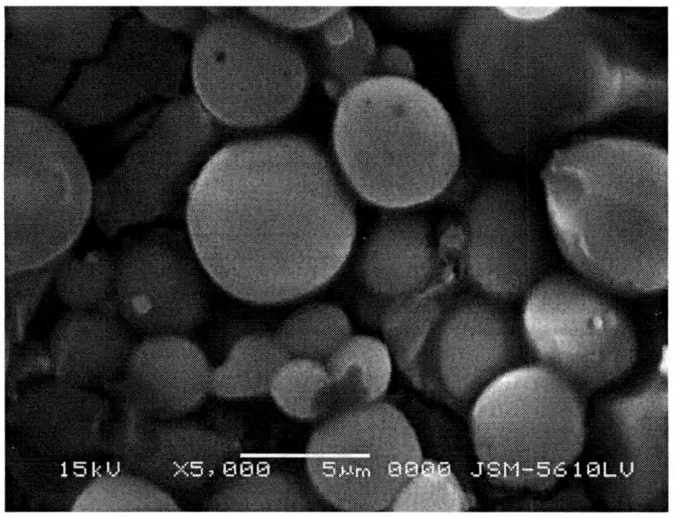
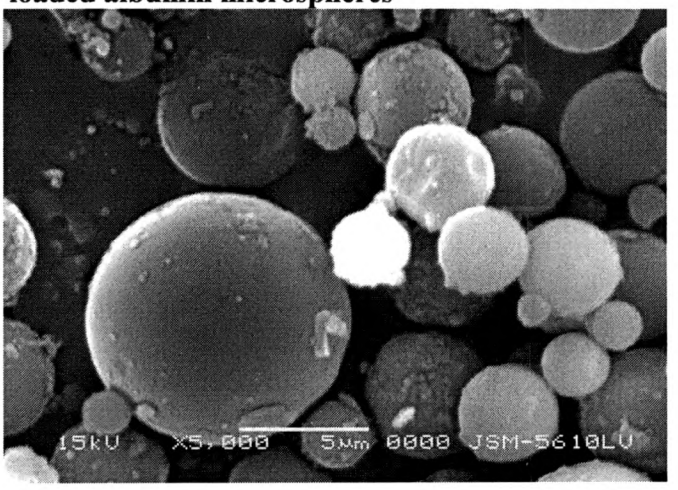


Figure 5.43: Scanning electron micrograph of celecoxib loaded albumin microspheres



5.10.4.5 FTIR

Figures 5.44 show the FTIR spectra of bovine serum albumin. It shows a typical FTIR spectrum of a protein with several amine and amide bands. The amide-I band (conjugation of –NH deformation mode with C=O and C=N stretching modes) is present at 1654.8cm^{-1}. The amide–II band due to the N-H bending vibrations is present at 1542.9cm^{-1} The presence of an absorption band at 3301.9cm^{-1} is due to the –NH stretching frequency of the amine group. The presence of a band at 2958.6cm^{-1} is due to the –C-H stretching vibrations.

Figures 5.45 and 5.46 shows the FTIR spectra of formaldehyde and glutaraldehyde crosslinked microspheres respectively. Figure 5.47 shows the FTIR spectrum of microspheres prepared by thermal denaturation.

The absence of the absorption band at 1700cm^{-1} in the microspheres crosslinked using formaldehyde or glutaraldehyde indicate that the microspheres are free from the residual formaldehyde or glutaraldehyde.

The absence of the characteristic bands of celecoxib in the FTIR spectrum of the microspheres reveals that celecoxib is entrapped in the microspheres and is not present in the free form.

Figure 5.44: FTIR spectrum of bovine serum albumin

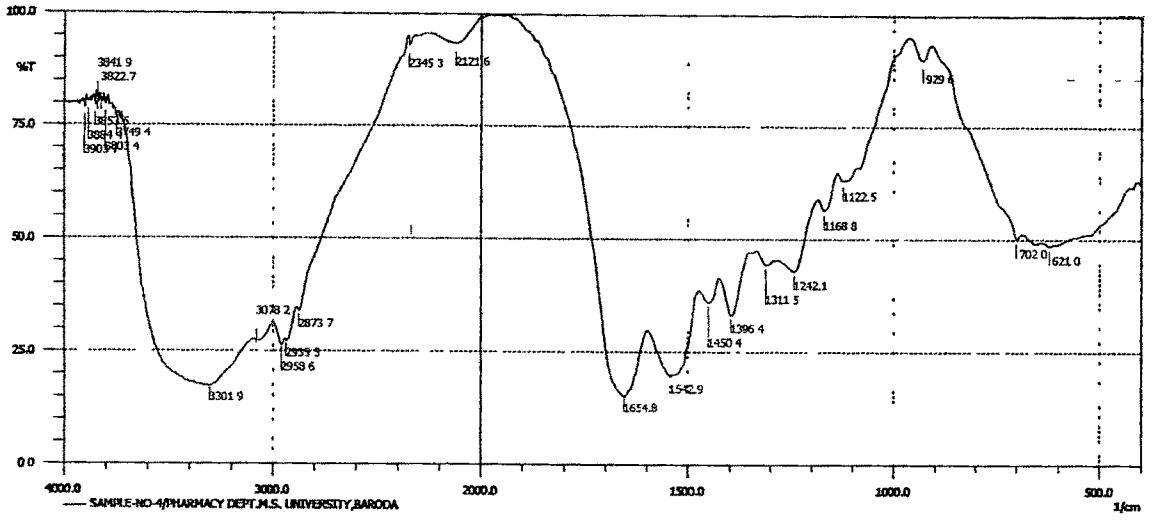


Figure 5.45: FTIR spectrum of formaldehyde crosslinked albumin microspheres

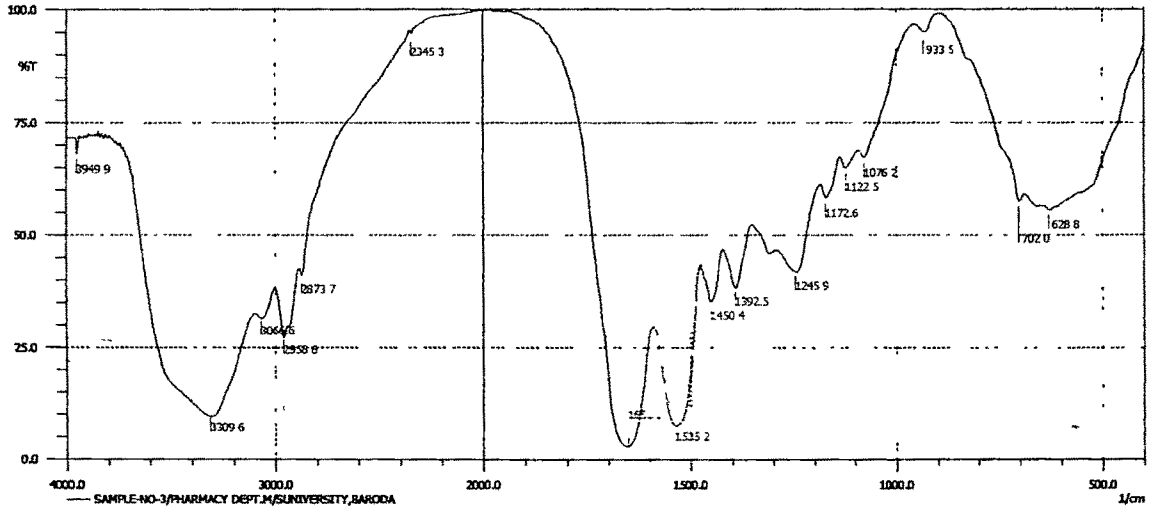


Figure 5.46: FTIR spectrum of glutaraldehyde crosslinked albumin microspheres

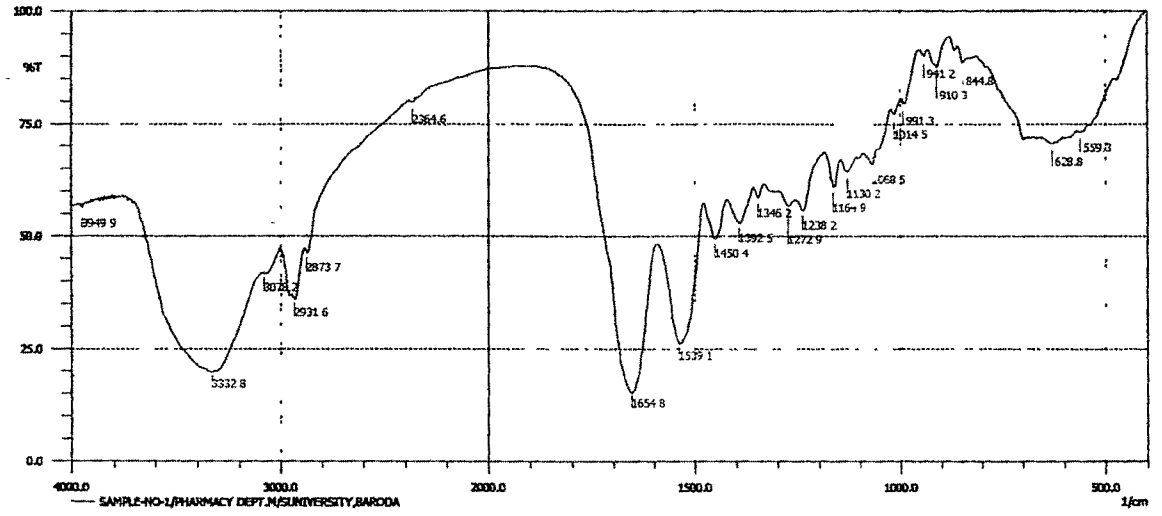
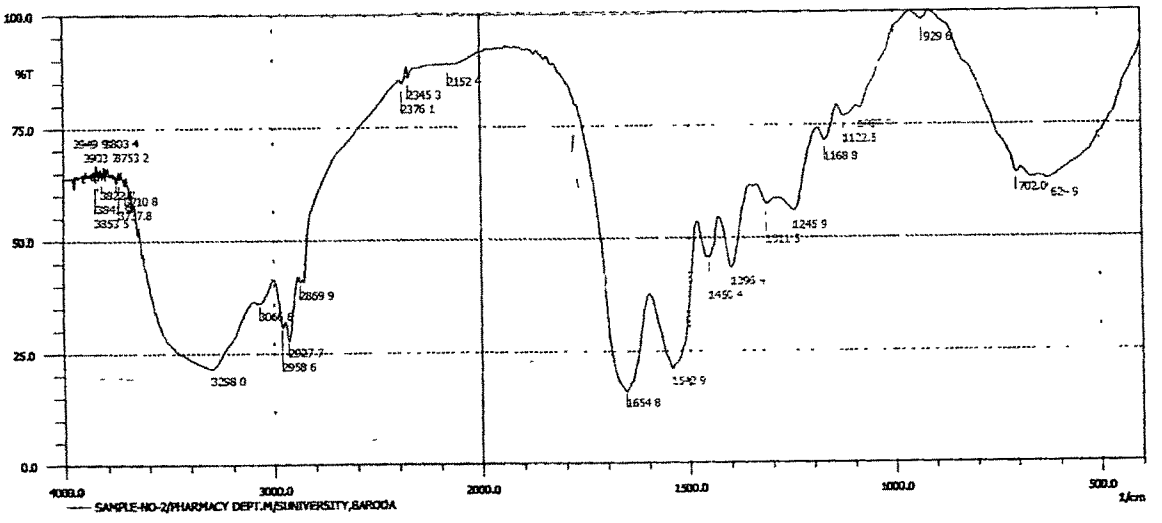


Figure 5.47: FTIR spectrum of albumin microspheres prepared by thermal denaturation



5.10.5 Solid lipid nanoparticles

Celecoxib loaded solid lipid nanoparticles were prepared using the lipid glycerol behenate and Poloxamer 407 as a surfactant. The nanoparticles were prepared by hot melt homogenization technique. The influence of factors such as concentration of Poloxamer 407, homogenization pressure and cycle number, concentration of lipid and celecoxib on the entrapment efficiency and the size of nanoparticles was studied.

5.10.5.1 Entrapment efficiency and particle size

For determination of amount of celecoxib entrapped in the solid lipid nanoparticles, the nanoparticle dispersion was extracted with dichloromethane in which celecoxib as well as the lipid are soluble. The extract was then evaporated to dryness and the residue was dissolved in 0.1N sodium hydroxide which selectively dissolved celecoxib and not the lipid. From the amount of celecoxib present in the nanoparticles, the entrapment efficiency was determined as shown in section 5.4. The effect of the different variables on the entrapment efficiency and particle size of the nanoparticles is discussed in the following sections:

5.10.5.1a Effect of concentration of lipid

Three different lipid concentrations were used in order to study the effect of the lipid concentration on the entrapment efficiency and particle size. Table 5.80 shows the effect of lipid concentration on the entrapment efficiency and particle size of the nanoparticles.

Table 5.80: Effect of concentration of lipid (compritol) on the entrapment efficiency and particle size

Batch code	Concentration of lipid (%w/v)	% Entrapment efficiency	Particle size (nm)
SLN-1	2	90.60±1.56	235±1.00
SLN-2	5	98.75±0.81	257±2.64
SLN-3	8	98.23±0.94	528±1.73

It can be seen from table 5.80 that with an increase in the lipid concentration from 2% to 5% there is an increase in the entrapment efficiency from 90.60% to 98.75%. With further increase in the lipid concentration to 8%, there is no significant increase in the entrapment efficiency. Hence 5% was selected as an optimum lipid concentration for preparation of the nanoparticles. The lower concentration (2%w/v) of lipid might not be sufficient for complete entrapment of celecoxib and hence higher entrapment efficiencies are obtained for batches SLN-2 and SLN-3 which were prepared using higher lipid concentrations. However, as celecoxib is a lipophilic drug and is completely soluble in the molten lipid, high entrapment efficiency (>90%) was obtained for all the batches.

There is a significant influence of the lipid concentration on the particle size of the nanoparticles. With an increase in the lipid concentration there is an increase in the particle size. This may be due to decrease in the effective surfactant (Poloxamer 407) concentration required to minimize agglomeration and particle growth. The results are in concurrence with the previous reports in which increase in the lipid content in the formulation over 5-10% led to the formation of particles with broader distribution and include microparticles also (Siekmann and Westesen, 1994). The solid lipid nanoparticles prepared using 5% compritol had higher entrapment efficiency and lower particle size. Hence 5%w/v was selected as an optimum concentration of lipid for further studies.

5.10.5.1b Effect of concentration of celecoxib

In order to study the effect of concentration of celecoxib on the entrapment efficiency and particle size, four different concentrations of celecoxib with respect to the concentration of compritol were used. Table 5.81 shows the effect of concentration of celecoxib on the entrapment efficiency and particle size of the nanoparticles.

Table 5.81: Effect of concentration of celecoxib on the entrapment efficiency and particle size of the nanoparticles

Batch code	Concentration of celecoxib(%w/w)	% Entrapment efficiency	Particle size (nm)
SLN-4	2	95.4±1.51	250±1.73
SLN-2	4	98.75±0.81	257±2.64
SLN-5	6	98.25±1.62	260±1.73
SLN-6	8	84.62±1.89	320±2.00

It is evident from table 5.81 that there is a significant influence of the concentration of celecoxib on the entrapment efficiency. With an increase in the concentration of celecoxib from 2% to 4% of the lipid, there is an increase in the entrapment efficiency from 95.4% to 98.75%. With further increase in celecoxib concentration to 6%, a decrease in entrapment efficiency was observed. When celecoxib concentration was increased to 8% there is a drastic reduction in the entrapment efficiency. With an increase in the celecoxib concentration, there is a reduction in the effective lipid concentration for entrapment of celecoxib. Thus, there is an increase in the unentrapped drug with an increase in the celecoxib concentration above a critical saturation point of the lipid.

There was an increase in the particle size with an increase in the celecoxib concentration. This increase is highly pronounced when celecoxib concentration is

increased from 6% to 8%. The reason behind this finding may be the increase in the size of the microdrops of the o/w emulsion at high drug loads leading to an increase in the size of the nanoparticles.

5.10.5.1c Effect of concentration of surfactant

The effect of concentration of Poloxamer 407 on the entrapment efficiency and particle size is shown in table 5.82.

Table 5.82: Effect of concentration of Poloxamer 407 on the entrapment efficiency and particle size of celecoxib loaded solid lipid nanoparticles

Batch code	Poloxamer 407 concentration (%w/v)	% Entrapment efficiency	Particle size (nm)
SLN-7	1%	97.68±1.35	902±2.00
SLN-2	3%	98.75±0.89	257±2.64
SLN-8	5%	98.10±1.08	255±2.64

Three different concentrations of Poloxamer 407 were used for the preparation of solid lipid nanoparticles. It is evident from table 5.82 that with an increase in the Poloxamer 407 concentration from 1% to 3% there is a significant reduction in the particle size of the nanoparticles. With an increase in the surfactant concentration, there is a reduction in the interfacial tension between the aqueous and the lipid phase which results in the formation of smaller droplets in the emulsion, which upon cooling results in nanoparticles with a smaller particle size. High surfactant concentrations effectively stabilize the particles formed by forming a steric barrier on the particle surface and thereby protect the particles from coagulation. With further increase in the Poloxamer 407 concentration to 5% there is no significant effect on the particle size. Thus 3% w/v was selected as an optimum concentration of Poloxamer 407 for the preparation of nanoparticles. There is no significant

influence of the Poloxamer 407 concentration on the entrapment efficiency of the nanoparticles as can be seen from the table 5.82.

5.10.5.1d Effect of homogenization pressure and number of cycles

After preliminary trials, the homogenization pressures in range of 7000 psi and 13000 psi which produced low particle size were considered for the study. Homogenization pressure and cycle number were found to have profound influence on the final size of the nanoparticles in dispersion. Three different homogenization pressures were selected for the study. Table 5.83 shows the effect of homogenization pressure and number of homogenization cycles on the entrapment efficiency and the particle size of the nanoparticles.

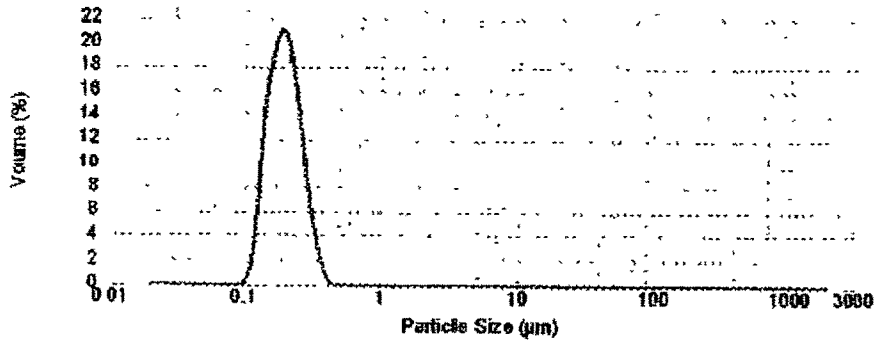
Table 5.83: Effect of homogenization pressure and number of cycles on the entrapment efficiency and particle size of the celecoxib loaded solid lipid nanoparticles

Batch code	Homogenization pressure (psi)	Number of cycles	%Entrapment efficiency	Particle size (nm)
SLN-9	7000	1	98.52±1.34	950±1.73
SLN-10	7000	2	97.84±0.70	835±1.00
SLN-11	7000	3	98.17±1.56	604±3.00
SLN-12	7000	4	97.50±2.03	590±2.64
SLN-13	10000	1	98.75±1.94	820±2.00
SLN-14	10000	2	99.36±0.27	605±2.64
SLN-2	10000	3	98.75±0.89	257±2.64
SLN-15	10000	4	98.57±1.91	334±2.00
SLN-16	13000	1	97.80±1.49	610±3.46
SLN-17	13000	2	98.63±1.73	466±3.60
SLN-18	13000	3	99.58±0.23	572±3.00
SLN-19	13000	4	98.67±1.36	568±2.00

At a homogenization pressure of 7000 psi, there is a decrease in the particle size with an increase in the number of cycles upto 4 cycles. When homogenization

pressure was increased to 10000 psi, the decrease in the particle size was observed upto 3 cycles after which the particle size increased. At 13000 psi, there was a decrease in the particle size upto 2 cycles after which the particle size increased. Table 5.83 shows that the lowest particle size was obtained for the nanoparticles prepared at 10000 psi and 3 cycles. Thus, these conditions were selected as optimum parameters for the preparation of the nanoparticles. Homogenization leads to development of cavitation forces which breaks down the particle structure to smaller ones. However, there is an optimum pressure and homogenization time upto which the lipid nanoparticles undergoes decrease in particle size. Above the optimum homogenization conditions, the excess cavitation forces and exposure of particles to these conditions for longer time leads to the particle aggregation. At higher homogenization pressures, the kinetic energy of the system increases resulting in particle collision and thereby the coagulation. The higher particle collisions also distort the surfactant film coating the particle surface and enhance particle aggregation. Hence an increase in the homogenization pressure as well as number of homogenization cycles beyond the optimum leads to aggregation of the nanoparticles and hence a drastic increase in the particle size is observed. Figure 5.44 shows the particle size distribution of the optimized batch SLN-2 prepared using 5% compritol, a homogenization pressure of 10000psi/3 cycles and drug loading of 4% and Poloxamer 407 concentration of 5%. It is evident that the particle size of the nanoparticles is monodisperse.

Figure 5.48: Particle size distribution of celecoxib loaded solid lipid nanoparticles



5.10.5.2 In-vitro drug release

The in-vitro drug release study of the optimized batch SLN-2 was performed by dialyzing the nanoparticles dispersion against the dissolution medium (phosphate buffer pH-7.4 with 2.0% tween-80). Table 5.84 and figure 5.49 shows the in-vitro release profile of celecoxib from the solid lipid nanoparticles.

Table 5.84: In-vitro release of celecoxib from solid lipid nanoparticles

Time(h)	Cumulative % release	
	Celecoxib	SLN-2
0.5	65.73±1.82	11.46±1.20
1	86.28±1.71	14.52±1.30
2	99.05±0.37	18.91±0.16
4		25.88±1.02
8		37.47±1.87
24		45.6±1.31
48		66.52±1.76
72		75.3±1.16
120		84.7±1.94
168		95.03±2.52

Figure 5.49: In-vitro release of celecoxib from solid lipid nanoparticles

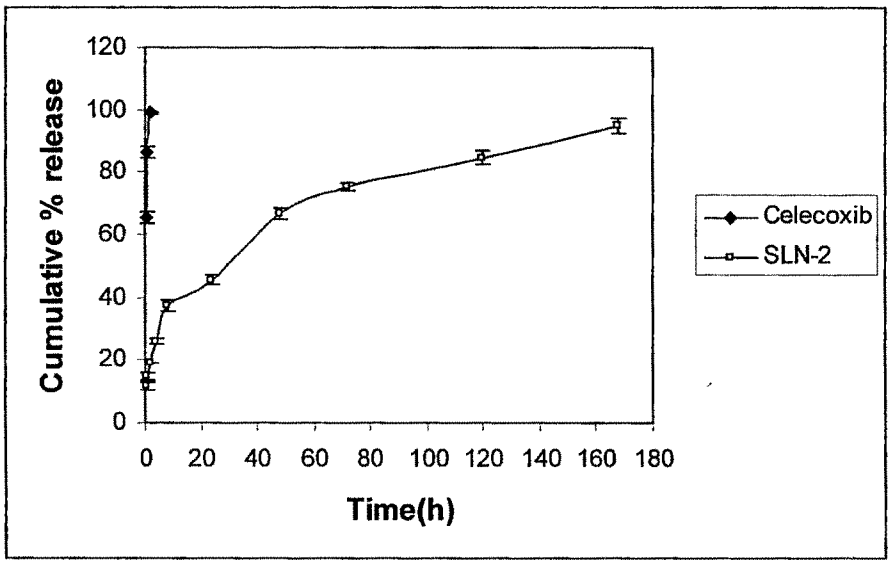


Table 5.85: Release kinetic parameters of celecoxib loaded solid lipid nanoparticles

r^2				n(peppas model)	K(peppas model)
Zero order	Higuchi	First order	peppas		
0.858	0.957	0.984	0.993	0.368	0.101

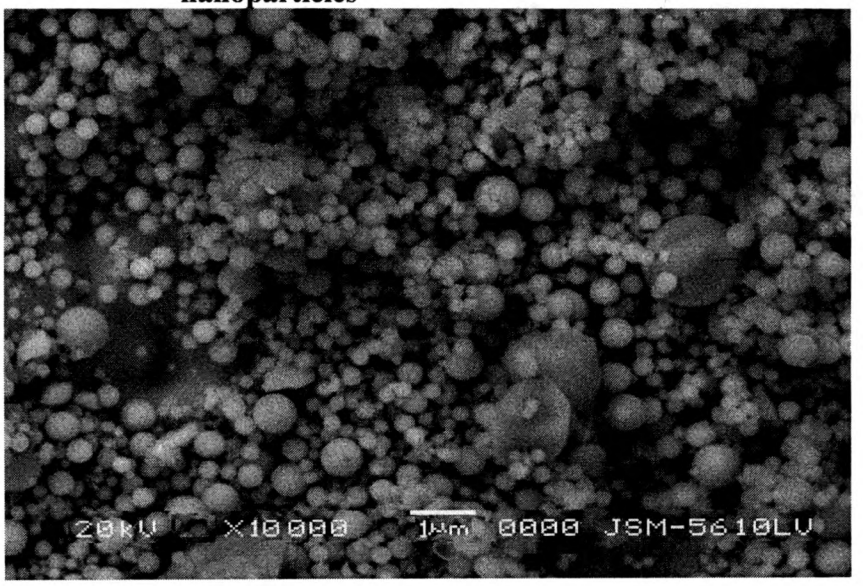
It can be seen from the data that the nanoparticles exhibit sustained release properties. There is a slight burst release. It can be seen from the figure 5.49 that the sustained release is not due to the barrier imposed by the dialysis bag. Dialysis of the plain celecoxib indicated that over 99% is released in about 2 hours. In contrast, celecoxib from the solid lipid nanoparticles exhibit sustained release (about 95% of celecoxib is released in 7 days) property.

To examine the kinetics of drug release mechanism, the release data were fitted to models representing zero order, first order and Higuchi's square root of time (Sankar and Mishra, 2003) and Korsemeyer-peppas model. The coefficient of correlation values are shown in table 5.85. The higher r^2 value obtained for the korsemeyer-peppas model indicates that the release of celecoxib from the solid lipid nanoparticles can be best explained by the korsemeyer-peppas model. The value of the release exponent n is less than 0.5, which indicates that the mechanism of celecoxib release from solid lipid nanoparticles is quasi fickian. The very low value of the release rate constant K indicates that there is a sustained release of celecoxib from the solid lipid nanoparticles.

5.10.5.3 Scanning electron microscopy

The scanning electron microscopy studies reveal that the surface of the microspheres is smooth and the particle size of the nanoparticles is fairly uniform.

Figure 5.50: Scanning electron micrograph of celecoxib loaded solid lipid nanoparticles



5.11 Stability studies

The optimized batches of each formulation were subjected to stability studies. Each formulation was stored in amber colored vials at room temperature for six months. The change in the % entrapment efficiency and particle size of each formulation was determined after 6 months.

Table 5.86: Stability study of the formulations

Formulation	Batch code	% Entrapment efficiency		Particle size	
		Initial	RT-6 months	Initial	RT- 6 months
Celecoxib loaded gelatin microspheres	G-4	86.82	85.96	20.51μm	20.83μm
Rofecoxib loaded gelatin microspheres	R-GM8	79.4	76.48	24.38μm	23.62μm
Valdecoxib loaded gelatin microspheres	V-GM6	95.4	95.17	22.47μm	22.80μm
Celecoxib loaded chitosan microspheres	D	90.14	88.73	8.65μm	9.04μm
Rofecoxib loaded chitosan microspheres	R-CM7	92.30	92.18	18.84μm	20.52μm
Valdecoxib loaded chitosan microspheres	V-CM7	87.48	86.55	9.06μm	9.73μm
Celecoxib loaded albumin microspheres	C-GA6	90.63	87.42	5.46μm	6.84μm
Rofecoxib loaded albumin microspheres	R-AMS11	89.17	88.36	6.55μm	6.38μm
Valdecoxib loaded albumin microspheres	V-AMS12	79.43	76.95	7.88μm	8.46μm
Celecoxib loaded solid lipid nanoparticles	SLN-2	98.75	96.32	257 nm	265nm

The results of the short term stability study of the optimized formulations indicate that the prepared formulations are highly stable. There is no significant change in the drug entrapment or the particle size of the microspheres on storage at room temperature for 6 months. All the formulations are in powder form which accounts for their excellent stability.

5.12 References

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