Research Article

Design and Development of Etoricoxib Loaded Nanoparticles for the Effective Treatment of Rheumatoid Arthritis

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Abstract

Background: Various conventional therapies for the management of Rheumatoid Arthritis (RA) are commercially available and generally used but these are associated with some various side effects. Because use of the existing or conventional therapies has its own set of limitations and risks of in terms of both safety and efficacy hence various novel approaches can be safely employed to treat this disease. **Objective**: To overcome the side effect and limitation of conventional drug therapy we aimed to prepare the surface modified novel drug delivery systems for efficient management of rheumatoid arthritis and to minimize the dose error problem. **Material and methods:** In the present work we have tried to explore the potential of the targeted nanoparticulate system for encapsulation and the delivery of selective COX-2 inhibitor etoricoxib. The BSA nanoparticles were prepared by desolvation method and the effect of various formulation and process variables on the particle size and drug entrapment efficiency was studied. **Results and Conclusion:** The results suggested that these variables influenced the shape, size, size distribution, and encapsulation efficiency of the nanoparticles. It could be seen that the diameter of nanoparticles decreased with the increasing content of BSA up to 0.2% concentration of BSA (w/v). From this study it may be concluded that development of folate-targeted therapeutic agents for guided intervention into arthritis may further enhance its site-specific drug delivery to activated macrophages at inflamed joints in RA and may possibly used as sustained drug delivery system in rheumatoid arthritis.

***Keywords*:** Rheumatoid Arthritis; COX-2 inhibitor; Etoricoxib; Site-specific drug delivery; Nanoparticles; Targeted nanoparticulate system

Introduction

The Global Burden of Disease 2016 study indicates that depressive disorders (major depressive disorder and dysthymia) now account for the third largest share of the world's burden of disease in terms of years lost to disability. Rheumatoid arthritis is also a major contributor to global disability. Rheumatoid arthritis (RA) is known as an inflammatory, chronic disease of joint with autoimmune basis and is mainly characterized by autoantibodies against immunoglobulin G (which is called rheumatoid factor [RF]) and anticitrullinated protein antibodies. In cases of incomplete treatment implementations, RA can result in joint harms and disability (Abbasi et al., 2019). These conditions affects joints and the surrounding tissues, as well as the connective tissue of the skin, bones, and muscles (Nogueira et al., 2015; Aletaha and Smolen, 2018).

The first recognized description of Rheumatoid arthritis was in 1800 by the French physician Dr Augustin Jacob Landre-Beauvais (1772-1840). The name "Rheumatoid arthritis" itself was coined in 1859 by British rheumatologist Dr Alfred Baring Garrod. Rheumatoid arthritis (RA) is a chronic and progressive autoimmune disease of unknown etiology, characterized by synovial inflammation as well as progressive destruction of cartilage and bone resulting in gradual immobility (Harrish et al., 1990).

RA can be mild, moderate, or severe. However, no matter what is the age of the person or severity, RA may seriously impede a person's ability to work and participate in other activities of daily living. In general, the younger a person is when he or she develops RA, the more rapidly that disease progresses. About 10% of people with the disease become severely disabled. In addition, life expectancy may be shortened by about 3 to 7 years, and those with severe forms

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of RA may die 10-15 years earlier than expected due to possible life-threatening complications.

RA is the most common form of chronic inflammatory arthritis that may affect many tissues and organs, but principally attacks sensorial joints. The process produces an inflammatory response of the symposium (synovitis) secondary to hyperplasia of synovial cells, excess synovial fluid, and the development of pannus in the synovium (McInnes and Schett, 2011). The pathology of the disease process often leads to the destruction of articular cartilage and ankylosis of the joints. Rheumatoid arthritis can also produce diffuse inflammation in the lungs, pericardium, pleura, and sclera, and also nodular lesions, most common in subcutaneous tissue under the skin. In rheumatoid arthritis, multiple joints are usually, but not always, affected in a symmetrical pattern. The "rheumatoid factor" is an antibody that can be found in the blood of 80% of people with rheumatoid arthritis. About 1% of the world's population is afflicted by rheumatoid arthritis, women three times more often than men. Onset is most frequent between the ages of 40 and 50, but people of any age can be affected. It can be a disabling and painful condition, which can lead to substantial loss of functioning and mobility (Majithia et al., 2007). RA characterized by both local systemic inflammation of both internal organs joints with elevated plasma concentration of pro inflammatory cytokines likes IL-6,IL-1b,TNF-alpha and other proteins (Figure 1).

The goals of treatment of RA are to reduce joint pain and swelling, relieve stiffness and prevent joint damage. Evaluation by a rheumatologist for the development and monitoring of a treatment plan is required in most people with RA. Treatment plans often include a combination of rest, physical activity, joint protection, use of heat or cold to reduce pain, and physical or occupational therapy. Maintains a healthy body weight and maintain a physical activity plan (i.e. Arthritis Foundation Exercise Program or Arthritis Foundation Aquatic Program) (Aletaha and Smolen, 2018).

Drug plays a very important role in the treatment of RA. Many people with RA take nonsteroidal anti-inflammatory drugs (NSAIDs) to help reduce joint pain, stiffness and swelling. Low doses of corticosteroids such as prednisone may also be used to relieve joint pain, stiffness and swelling and to reduce the risk of joint swelling. Various conventional therapies for the management of RA are commercially available and generally used but these are associated with some various side effects. NSAIDS (Non-Steroidal Anti-inflammatory Drugs), DMARDs, corticosteroids, and various biological agents do not attain desired bioavailability due to short life span, easily metabolized by the enzymatic system of the body or sometimes may be the rapid clearance before reaching therapeutic concentration. Prolong application of NSAIDS for RA may cause severe GIT ulcers, nephrotoxicity etc. Steroidal drug used for longtime in high dose may cause weight gain, osteoporosis, increase in blood pressure and high blood sugar etc. These steroids are also having a short onset of action while DMARDs are available to acting potentially but it takes severe weeks or months to demonstrate a clinical effect. So, none of these medications cures the disease completely and they all have the potential to cause significant adverse effects (Valerio et al., 2021).

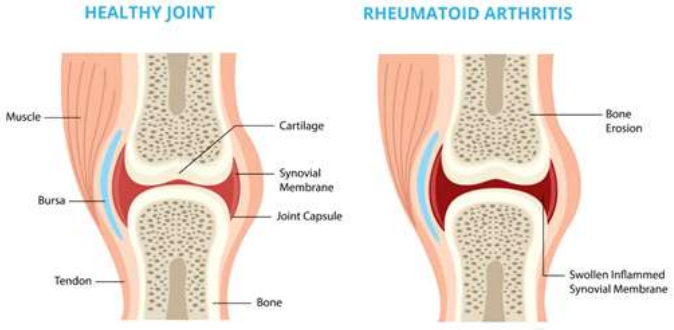


Figure 1. Difference between healthy joint and arthritic joint (Firestein, 2003)

Because use of the existing or conventional therapies has its own set of limitations and risks of in terms of both safety and efficacy hence various novel approaches can be safely employed to treat this disease. To overcome the side effect and limitation of conventional drug therapy we can prepare the surface modified novel drug delivery systems for efficient management of rheumatoid arthritis and to minimize the dose error problem (Padjen et al., 2020). Choice of drugs in this therapy for the effective management of rheumatoid arthritis based on the fact that drug within functionalized carrier system is much as effective treatment to cure any chronic systemic inflammatory disease. This project solely based on therapy with NSAIDs drug. The model drug loaded nanocarrier provides efficient management as effective to reach required bioavailability, low cytotoxicity and minimum side effects over the conventional single drug therapy.

Nanocarrier based drug delivery system like nanoparticle offers many advantages over conventional or free drug administration. Remarking, nanoparticles are capable of: (i) encapsulate more drug and protect drugs from degradation (physical/chemical/biological) or deactivation before to reaching target site in vivo, (ii) improve targeting over free drugs via presentation of tissue-specific targeting ligands, (iii) offers controlled drug release by altering nanoparticle composition, from all these reasons, nanoparticles hold the potential to be the ideal drug delivery carrier (Anita et al., 2021).

Folate conjugated nanoparticles offers a targeted delivery via selective cellular markers can potentially increase the efficacy and reduce the toxicity of therapeutic agents by acting on the β-folate receptor which is overexpressed in the rheumatoid arthritis. It offers potential advantages over conventional non-targeted therapy, most notably a reduction of access of drug to non-target sites and reduces the side effects of drug due to decreased delivery to non-target organs. These nanoparticles have been intensively investigated as a novel drug carrier due to their extensive advantages, such as good biocompatibility, biodegradability, and nontoxic properties Therefore, chitosan nanoparticles are needed in improving the efficiency of drug delivery system.

The objective of current study is to design carrier systems in which folate engineered nanoparticles shall be encapsulate the anti-rheumatic drug of choice. These folate conjugated nanoparticles assess for their in vitro release pattern as well as in vivo targeting potential for rheumatoid arthritis. Hence, it is envisaged to design a Novel approach which appears to be excellent candidate for management of rheumatoid arthritis and shows new reflection on the impact of targeting on the rheumatic sites as folate conjugated nanoparticles.

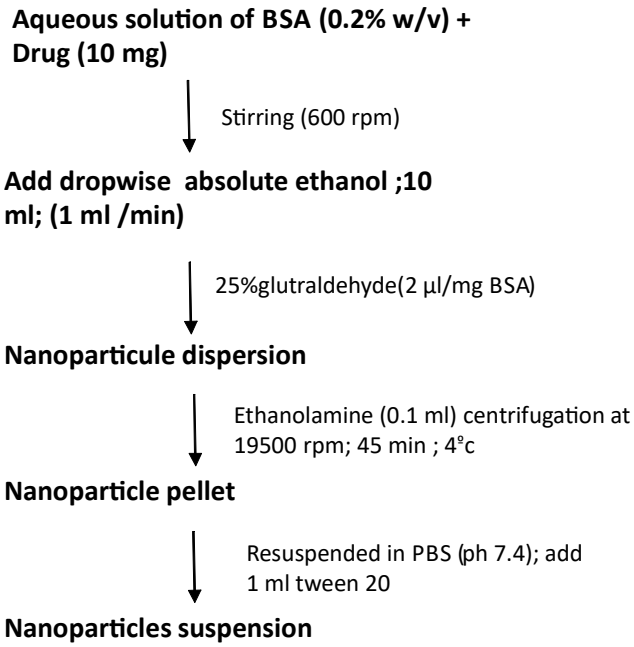
Materials and Methods

Materials

The drug etoricoxib was obtained as a gift sample from Torrent research center, Ahmedabad, India. Bovine serum albumin (BSA) was purchased from spectrochem (Mumbai). Folic acid was purchased from Himedia (Mumbai). All other solvents and reagents were of analytical and HPLC grade.

Preparation of Albumin Nanoparticles

BSA nanoparticles were prepared by desolvation method with some modification (Esim et al., 2021). BSA (0.05 to 0.2%w/v) was weighed and dissolved in 5 ml of distilled water with constant stirring. The drug (5 to 20 mg) was also added to this aqueous BSA solution. The pH of this aqueous dispersion was adjusted towards the isoelectric point of BSA (pI= 5.5) using 0.1 N HCl .Ten ml of absolute alcohol was added drop wise (with predetermined rate) under the continuous stirring till the solution became turbid. Coacervates thus obtained were hardened for 2 hr with 25% glutraldehyde (0.1 to 0.4µl/mg BSA). Ethanolamine (0.1 ml) was also added to block the unreactive aldehyde groups. Afterwards the undissolved drug and large aggregates were separated by centrifugation at low rpm (~2000) for 5 min. The nanoparticles suspension was separated and centrifuged at 19500 rpm for 45 min. at 4°C (Remi INDIA). The supernatant was removed and the pellet was resuspended in PBS (pH 7.4; 0.1 M). One ml tween 20 was also added to this to stabilize the preparation. The whole procedure is shown in flow diagram (Scheme 1).



Scheme 1. Flow diagram of preparation of BSA Nanoparticles

Ligand Conjugation

After preparation of nanoparticles using optimized variables folic acid was conjugated at the surface of preformed NPs of BSA according to the method reported by Zhang *et al.,* (2010).

(a) Activation of folic acid

N-Hydroxysuccinimide ester of folic acid (NHS-folate) was prepared. Folic acid (100 mg) was dissolved in solvent system of 2 ml of dry dimethyl sulfoxide and 50 µl of triethylamine. Subsequently *N*-hydroxysuccinimide (50 mg) in the presence of dicyclohexylcarbodimide DCC (90 mg) was added and the dispersion was stirred over night at room temperature. The by-product, dicyclohexylurea was removed by filtration. The dimethyl sulfoxide solution was then concentrated under reduced pressure and heating, and NHS-folate was precipitated in diethylether. The product, NHS-folate was washed several times with anhydrous ether, dried in vacuum, and yielded as yellow powder.

(b) Conjugation of activated folate to drug loaded NPs

NHS-folate (50 mg) prepared above was dissolved in 1.0 ml of dimethyl sulfoxide and added slowly to the stirring BSA-NPs suspension (2 ml, pH was adjusted to 10 using 1M carbonate/bicarbonate buffer). After stirring for 45 min at room temperature, the reaction mixture was passed down a Sephadex G-50 column to separate the folate-conjugated BSA-NPs from unreacted folic acid and other by-products. The folate-conjugated BSANPs eluted in the void fraction.

The suspension was centrifuged and the pellets were redispersed to the original volume in PBS (pH 7.4).

In vitro characterization of formulations

The optimized drug loaded nanoparticulate formulation (ETX-NPs) and folate conjugated drug loaded nanoparticles (f-ETX-NPs) were subjected to *in vitro* characterization for particle size, size distribution, shape and surface morphology, and encapsulation efficiency.

IR spectroscopy

Drug loaded nanoparticles (ETX-NPs), activated folic acid and folate conjugated drug loaded nanoparticles (f-ETX-NPs) were scanned as KBr pellet on IR spectrophotometer (Perkin Elmer 3600, USA) and their IR spectrums were obtained. The IR spectrum are shown in Figure 2. The characteristic peakss are recorded in Table 1.

Table 1. Interpretation of characteristic bands in IR spectrum of f-ETX-NPs

Peaks (cm^{-1})	Band assignment
3392.3	N-H stretching
2977.4	C-H stretching (Ar.)
1740.2	C=O stretching
1645.2	N-H bending
1398.1	O-H bending

Determination of Shape and Surface morphology

The shape as well as surface morphology of the prepared nanoparticles was investigated by using transmission and scanning electron microscopy (TEM and SEM).

TEM was performed using a Hitachi (H-7500) electron microscope. A drop of sample was placed on a carbon coated copper grid to leave a thin film on the grid. Before the film dried on the grid, the film was negatively stained with 1% phosphotungstic acid (PTA). A drop of staining solution was added on the film and the excess of the solution was drained off with a filter paper. The grid was allowed to air dry thoroughly and sample were viewed under transmission electron microscope and photographs were taken at suitable magnification (Figure 3).

SEM was performed using JSM 6100 (JEOL) electron microscope. The sample was prepared by taking lyophilized formulation on a double adhesive tape, which was stuck to an aluminium stub. The stub was then coated with gold. The sample was then randomly scanned and photograph was taken (Figure 4).

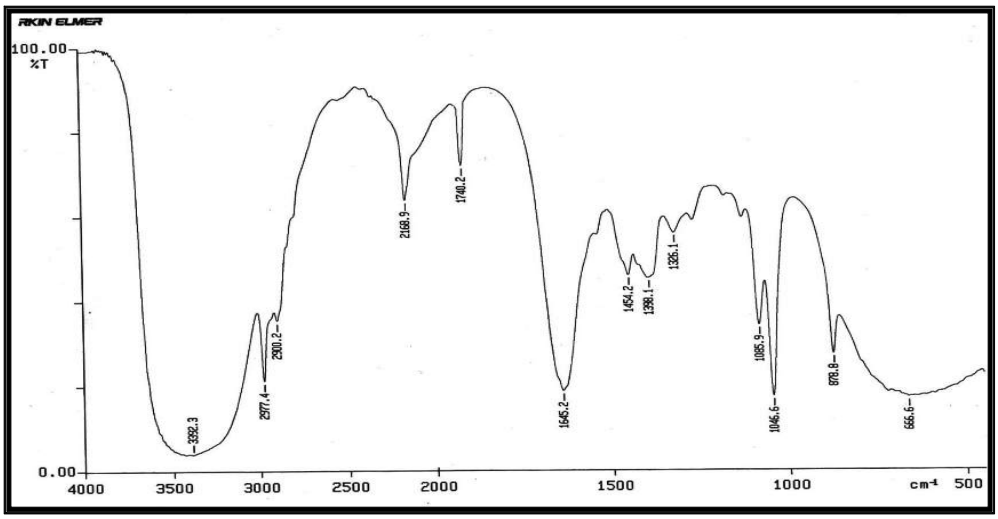


Figure 2. FT-IR spectrum of f-ETX-NPs

Table 2. Entrapment of Etoricoxib in 10 mg of NPs (n=3)

Drug added (mg)	Drug Entrapped (mg)	% Drug Entrapment	% Drug loading
5	2.03±0.19	40.6±3.2	20.3±2.18
10	4.23±0.42	42.3±3.7	42.3±4.36
15	4.24±0.51	28.2±2.8	42.4±4.40
20	4.30±0.62	21.5±3.1	43.0±4.42

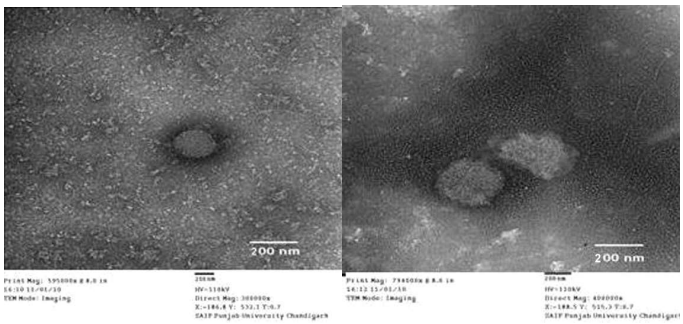


Figure 3. TEM images of nanoparticles (**A**) Drug-loaded nanoparticles; (**B**) Folate conjugated drug-loaded nanoparticles

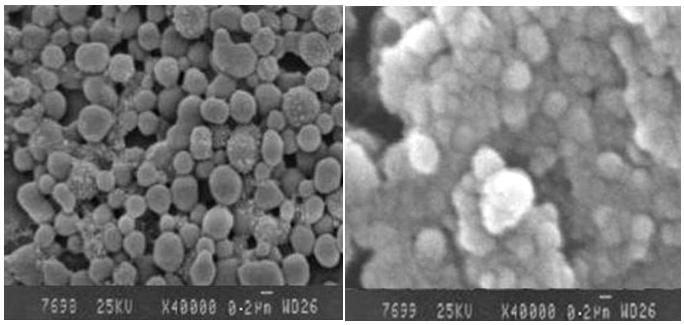


Figure 4. SEM images of nanoparticles (**A**) Drug-loaded nanoparticles; (**B**) Folate conjugated drug-loaded nanoparticles

Determination of particle size, PDI and zeta potential

Particle size

The average particle size and polydispersity index of the NPs were determined by photon correlation spectroscopy using a zetasizer (DTS ver.4.10 Malvern instrument, England). The sample of dispersion was diluted 1:9 v/v with deionized water and determined the particle size and polydispersity index was determined using Malvern zeta sizer. The average volume-mean particle size was measured after performing the experiment in triplicate.

Zeta potential of the nanoparticles

Each sample of the nanoparticles suspension was adjusted to a concentration of 0.05% (w/v) in filtered water in the case of zeta potential examination. The zeta potential was determined in triplicate for a single batch of nanoparticles and the results are recorded as the average of three measurements.

Encapsulation efficiency, drug loading capacity

The entrapment efficiency of nanoparticles was determined by measuring the difference between the total amount of drug (Etoricoxib) added in nanoparticle preparation and amount non-entrapped. For this a weighed amount of nanoparticles was suspended in 0.1 N hydrochloric acid to remove surface absorbed and unentrapped drug. Subsequently the nanoparticles were seperated by centrifugation and ruptured using 0.1 N hydrochloric acid containing 2% wt/vol. of pepsin and allowed to stand for 24 hours. The dispersion was centrifuged, filtered through 0.45 µm membrane filter and the absorbance of the resulting supernatant solution was measured at 233.5 nm on a Shimadzu-UV 1601 spectrophotometer (Shimadzu, Kyoto, Japan) to determine the amount of drug present in the nanoparticles.

Table 2 illustrates the % drug entrapment and % drug loading in the plain nanoparticulate formulation while the Table 3 illustrates the % drug entrapment and % drug loading in the folate conjugated nanoparticulate system.

***In vitro* drug release from the nanoparticles**

The *in vitro* drug release profile of nanoparticulate formulations was determined as follows. A weight amount of nanoparticles equivalent to 2.5 mg of etoricoxib were re-dispersed in phosphate-buffered saline (PBS, pH 7.4) and placed in a dialysis membrane bag with a molecular cut-off of 8 kDa. Then the NPs containing bag was tied and put into 100 mL of PBS (pH 7.4) medium. The entire system was incubated at 37±2C under stirring at 50 rpm. At designated time intervals, 0.5 ml of the release medium was removed and replaced with the same volume of fresh PBS solution. The amount of drug in the release medium was determined spectrophotometrically at 207 nm. All measurements were performed in triplicate (Table 4, Figure 5).

Result and discussion

The BSA nanoparticles were prepared by desolvation method and the effect of various formulation and process variables on

Table 3. Entrapment of Etoricoxib in 10 mg of ligand conjugated NPs (n=3)

Drug added (mg)	Drug Entrapped (mg)	% Drug Entrapment	% Drug loading
5	1.97±0.18	39.4±3.5	19.7±2.05
10	3.9±0.42	38.9±3.8	38.9±4.18
15	4.12±0.61	27.4±3.8	41.2±4.70
20	4.28±0.72	21.4±3.2	42.8±4.95

Table 4. Percentage cumulative drug release in PBS (pH 7.4) (n=3)

Time (hr)	Plain drug solution (%)	Drug loaded nanoparticles (%)	Ligand conjugated Drug loaded nanoparticles (%)
1	23.15±1.82	14.25±0.81	15.15±0.93
2	37.45±3.16	19.40±1.23	18.12±1.01
3	42.25±3.24	24.78±1.41	23.45±1.35
4	53.65±0.36	29.45±1.58	26.32±1.22
5	65.67±4.91	32.68±2.22	30.11±1.67
6	72.45±2.91	38.56±2.91	35.12±2.10
12	89.52±2.63	52.12±3.20	51.45±2.88
18	99.12±2.81	62.41±3.72	56.23±3.16
24	-	74.52±3.91	71.08±3.20
48	-	81.45±4.21	76.23±3.91
72	-	99.12±2.69	93.11±2.26
96	-	-	99.23±0.52
120	-	-	-

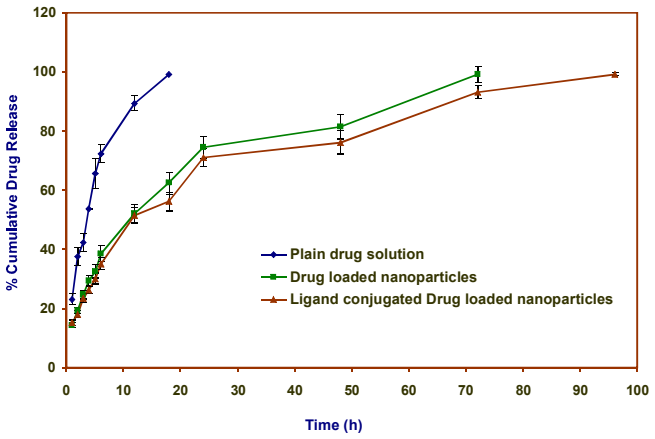


Figure 5. Percentage cumulative drug release Vs Time (hr)

the particle size and drug entrapment efficiency was studied. The results suggested that these variables influenced the shape, size, size distribution, and encapsulation efficiency of the nanoparticles. It could be seen that the diameter of nanoparticles decreased with the increasing content of BSA up to 0.2% concentration of BSA (w/v). This may be due to the repelling forces among the negative charged particles. On further increasing the BSA concentration the particle size was increased which may be attributed to the shielding function of additional surface charges. The mean particle size of BSA nanoparticles varied from 140±2.1 nm to 210±2.3 nm on varying the concentration of glutraldehyde from 1 µl to 4µl/mg (v/w) of BSA, this may be due to the additional decrease in the hydrophilic behavior of albumin induced by the enhanced cross linkage. At 2 µl/mg BSA ,glutraldehyde concentration, nanoparticles with maximum drug entrapment (39.7±1.38%) and minimum particle size (180±2.4 nm) were formed.

On varying the amount of etoricoxib from 5 mg to 20 mg the average particle size of BSA nanoparticles varied from 173±3.1 nm to 220±1.6 nm. The average size of nanoparticles was increased with increasing the drug concentration. Ten mg etoricoxib concentration was found to be optimum over which the maximum drug entrapment (42.3±0.74%) was achieved. On further increasing the drug concentration i.e. more than 10 mg, there was no significant increase in the drug entrapment efficiency of nanoparticles was observed.

On varying the stirring speed from 200 rpm to 800 rpm, the average particle size of BSA nanoparticles varied from 220±4.6 nm to 160±2.4 nm. At 600 rpm more uniform particles with maximum entrapment and optimized size are formed.

On varying the rate of ethanol addition from 0.5 ml/min to 2.0 ml/min the average particle size of BSA nanoparticles varied from 160±3.03 nm to 215±2.5 nm. This might be due to the fast albumin phase separation caused by the added ethanol. Quick desolvation leads to the formation of larger particles. The ethanol addition rate of 1.0 ml/min was found to be optimum for BSA nanoparticles because at this rate, the size of nanoparticles was optimum with good loading efficiency of 42.8±1.18%.

After preparation of nanoparticles, folic acid was conjugated at the surface of preformed NPs of BSA through amide bond between –COOH group of folic acid and $–NH_2$ group of albumin. The coupling of folic acid with nanoparticles was confirmed by comparing the IR spectrum of drug loaded NPs with that of folate conjugated drug loaded NPs.

IR spectrum of drug loaded BSA NPs showed peaks at 3428.6 cm^{-1} (N-H stretching) and 878.2cm^{-1} (S-S stretching) showing characteristic bands of BSA.The spectrum also shows peaks at 669.5 cm^{-1} (C-Cl Stretching vibration) a characteristic band for the drug. In FT-IR spectrum of drug loaded NPs conjugated with folic acid, the peaks at 1740.2 cm^{-1} and 1645.2 cm^{-1} represent –CO stretching (carbonyl group) and –NH bending (1°amine) respectively, indicating the formation of amide bond between carboxylic group of polymer and EDC (acting as cross-linking agent between the ligand and polymer) (Figure 2 and Table 1).

These prepared uncoupled and folic acid coupled BSA nanoparticles were characterized for shape and surface morphology, particle size, zeta potential and drug entrapment efficiency.

The TEM and SEM photomicrographs exhibit that NPs are spherical in shape but the surface of folic acid coupled NPs is less smooth as compared to their uncoupled NPs (Figure 3 and 4). The average size of uncoupled nanoparticles was found to be 180.5±3.2 nm, while the size of coupled nanoparticles was found to be 215.8±2.9. The increase in size could be due to the coupling of folic acid at the surface

of NPs. The zeta potential of NPs was decreased on coupling of folic acid to the surface of NPs. The decreased zeta potential may be due to the reaction of -NH_2 group of BSA and the –COOH group of folic acid.

The drug entrapment efficiency of coupled NPs was decreased to 38.17±1.4% as compared to uncoupled NPs (42.8±1.18%) (Table 2 and 3). This could be due to the slight leakage of drug from the outer most layer of NPs during coupling of folic acid to the nanoparticulate surface.

Optimized formulation was characterized for *in vitro* drug release study using dialysis bag method. Result shown in Table 4 and Figure 5 indicate that drug release from the optimized formulation was 99.12±2.69%, after 72 hrs while ligand coupled NPs exhibited 93.23±0.52% drug release in 72 hrs which is less than the uncoupled NPs, this could be due to the presence of an additional barrier layer of folic acid over the surface of NPs.

Conclusion

Present study may be summarized that by adjusting the different formulation and process variables an optimized formulation with desired particle size and entrapment efficiency may be prepared.

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