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Sustained release optimized formulation of anastrozole-loaded chitosan microspheres: in vitro and in vivo evaluation

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Abstract The purpose of this study was to develop sustained release formulation of anastrozole-loaded chitosan microspheres for treatment of breast cancer. Chitosan microspheres cross-linked with two different cross-linking agents viz, tripolyphosphate (TPP) and glutaraldehyde (GA) were prepared using single emulsion (w/o) method. A reverse phase HPLC method was developed and used for quantification of drug in microspheres and rat plasma. Influence of cross-linking agents on the properties of chitosan microspheres was extensively investigated. Formulations were characterized for encapsulation efficiency (EE), compatibility of drug with excipients, particle size, surface morphology, swelling capacity, erosion and drug release profile in phosphate buffer pH 7.4. EE varied from 30.4 ± 1.2 to 69.2 ± 3.2% and mean particle size distribution ranged from 72.5 ± 0.5 to 157.9 ± 1.5 μm. SEM analysis revealed smooth and spherical nature of microspheres. TPP microspheres exhibited higher swelling capacity, percentage erosion and drug release compared to GA microspheres. Release of anastrozole (ANS) was rapid up to 4 h followed by slow release status. FTIR analysis revealed no chemical interaction between drug and polymer. DSC analysis indicated ANS trapped in the microspheres existed in amorphous form in polymer matrix. The highest correlation coefficients (R^2) were obtained for Higuchi model, suggesting a diffusion controlled mechanism. There was significant difference in the pharmacokinetic parameters ($AUC_{0-\infty}$, Kel and $t_{1/2}$) when ANS was formulated in the form of microspheres compared to pure drug. This may be attributed to slow release rate of ANS from chitosan microspheres and was detectable in rat plasma up to 48 h which correlates well with the in vitro release data.

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1 Introduction

Breast carcinoma has become a major health problem over the past 50 years, affecting as many as one in eight women. Although there have been substantial developments in its treatment, approximately 25% of women with breast carcinoma will eventually die from the disease. Treatment of breast cancer has included efforts to decrease estrogen levels by the use of anti-estrogens. These agents act by the inhibition of enzyme aromatase, that catalyses the conversion of the androgens, androstenedione and testosterone, to estrogens (estrone and estradiol), the major route of estrogen synthesis in postmenopausal women and progestational agents [1, 2]. Anastrozole (ANS), chemically known as 2,2′-(5-((1H-1, 2,4-triazole-1-yl) methyl)-1,3-phenylene)-bis(2-methylpropanenitrile), is a potent new-generation nonsteroidal aromatase inhibitor of the triazole class, approved by the United States of Food and Drug Administration (USFDA) for the treatment of advanced breast cancer in post-menopausal women. ANS (Arimidex®) administered as 1 mg tablet by oral route once daily [3, 4].

For patients with advanced breast cancer and for adjuvant treatment of early breast cancer in postmenopausal women, the median duration of therapy should be prolonged, lasting for about 31 months [5, 6]. ANS is generally well tolerated in the majority of patients, the most common adverse events being gastrointestinal (GI) disturbances (incidence 39–43%). Hence to increase patient compliance, a sustained delivery system of ANS can be realized for treatment of breast cancer.

One of the technological resources used to improve the performance of drugs at the site of action is the use of therapeutic systems prepared using biodegradable polymers. Biodegradable polymers show increasing importance in the development of sustained release drug delivery system. Commonly used biodegradable polymers in the preparation of microspheres are poly(lactide-co-glycolide), poly(ε-caprolactone), chitosan, gelatin, albumin etc. Drug release rate can be controlled by selection of their molecular weight and copolymer compositions. Natural polymers, like polysaccharides are widely used in pharmaceutical applications due to there biocompatibility, biodegradability and low cost. Chitosan being a natural, biodegradable and biocompatible polymer is considered as a potential carrier for delivery of therapeutic agents. It has been used in the formulation of particulate drug delivery system to achieve sustained or controlled drug delivery [7].

The purpose of this research was to design the sustained release of anticancer agent, ANS using chitosan based drug delivery systems [8, 9]. Chitosan, a natural biopolyaminosaccharide, is obtained by alkaline deacetylation of chitin that is found widely in nature. It is metabolized by certain human enzymes, especially lysozyme and hence is considered as biodegradable polymer. It has attracted significant interest in recent years, is a weak base and is insoluble in water and organic solvents; however, it is soluble in dilute aqueous acidic solutions (pH 5.0–6.5), which can convert the glucosamine units into a soluble form $R\text{-}NH_3^+$ [10]. It gets precipitated in alkaline solution or with polyanions and forms gel at lower pH. Due to the easy availability of free amino groups in chitosan, it carries a positive charge and thus, in turn reacts with negatively charged surfaces/polymers [11, 12]. This principle has been used for the production of chitosan microcapsules and microspheres for sustained drug delivery. These microspheres are being investigated both for parenteral and oral drug delivery. Chitosan microspheres can be prepared by reacting chitosan with controlled amount of multivalent anion resulting in cross-linking between chitosan molecules [13]. The cross-linking may be achieved in acidic, neutral, or basic environments depending upon the method applied. Chitosan microspheres can be prepared by various methods such as cross-linking with anions, precipitation, complex-coacervation, modified emulsification and ionotropic gelation, precipitation-chemical cross-linking, glutaraldehyde (GA) cross-linking, thermal cross-linking etc. [14]. Out of these methods, chemical denaturation method is commonly used which involves denaturation of chitosan present in the inner phase of water/oil (w/o) emulsion. Many process parameters affecting characteristics of chitosan microspheres have been identified and the significance of the effect has been established. The chemical cross-linking method for preparation of chitosan microspheres involves emulsification followed by cross-linking with a suitable cross-linking agent [e.g. GA and tripolyphosphate (TPP)]. The degree of stirring (i.e., time and speed of stirring during emulsification) determines the size of dispersed droplets. By varying any one or both of these parameters, the size of droplets can be changed to obtain the product (i.e., chitosan microspheres) in the desired particle size range. The present investigation was intended to formulate and evaluate chitosan microspheres, which would deliver ANS for prolonged period of time. In order to stabilize chitosan microspheres, a crosslinking agent such as GA and TPP have been used and the effect of these were studied on the properties [% encapsulation efficiency (EE), size, surface morphology, % erosion, % swelling, in vitro release behaviour and in vivo behaviour in rats] of chitosan microspheres.

2 Materials and methods

2.1 Materials

Anastrozole (99.5% purity) was obtained as a gift sample from Sun Pharmaceuticals Pvt. Ltd., Baroda, India. Chitosan, medium molecular eight ($M_w = 1.336 \times 10^6$)—as reported in manufacturer's Certificate of Analysis (COA) with 85% degree of deacetylation (DDA) was purchased from Sigma-Aldrich Chemie, USA. GA and TPP were purchased from Sigma-Aldrich Chemie, USA. Span-80 and Tween-80 were procured form Spectrochem Pvt. Ltd., Mumbai, India. Acetonitrile (HPLC grade) was purchased from Merck Specialties Pvt. Ltd., Mumbai, India. Monomeric Column C_{18} (250 × 4.6 mm, 5 μm) was purchased from Grace Vydac®, USA. All other chemicals used were of analytical grade. Millipore water (Millipore®, Bedford, MA, USA) was used throughout the study.

2.2 Development of ANS analytical method

A sensitive and selective reverse phase high performance liquid chromatography (HPLC) method was developed and validated for the rapid quantification of ANS in micro quantities. The integrated HPLC system, LC-2010C_{HT} (Shimadzu, Kyoto, Japan) equipped with low pressure

quaternary gradient pump along with dual wavelength UV-detector, column oven and auto sampler has been used for the analysis. The chromatographic data was processed using LC solution 1.24 SP1 software. The chromatographic separation was achieved with Grace vydac® monomeric column C_{18} (250 × 4.6 mm, 5 μm) and mobile phase consisted of acetonitrile: phosphate buffer (10.0 mM, pH 3.0 ± 0.2 adjusted with dilute ortho phosphoric acid) in a ratio of 60:40 v/v. The analysis was performed at room temperature using a flow rate of 1.0 ml/min with a UV detection wavelength of 210 nm [15]. The column oven temperature was maintained at 25°C and samples were kept at 4°C. The total run time of the developed method was 12.0 min. The calibration curve was constructed over the range was 0.5–100.0 μg/ml with injection volume of 50.0 μl.

Validation of the developed chromatographic method was carried out to ensure performance of the method. The developed method was validated as per International Conference on Harmonization (ICH) for the parameter specificity, precision, recovery to assess reliability of the developed method. As part of method validation linearity was constructed over the range of 0.5–100 μg/ml for three different days. Inter-day and intra-day precision was evaluated at the three levels i.e. 1, 10 and 40 μg/ml of ANS. The recovery of the method was evaluated by spiking blank microsphere matrix. The recovery was evaluated at three level of concentration of 5, 10 and 15 μg/ml.

2.2.1 *Preparation of stock and standard solutions*

The stock solution of ANS reference standard was prepared in methanol (1 mg/ml). Secondary stock solution was prepared from primary stock solution by dilution with methanol. Calibration standard solutions were prepared in mobile phase by appropriate dilution of secondary stock solution to get the concentration of 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0 and 100.0 μg/ml. The calibration curve was constructed by plotting the peak area on ordinate as a function of ANS concentration on abscissa.

2.3 Formulation development

2.3.1 *Preparation of ANS microspheres*

The microspheres were prepared by modified single emulsion technique coupled with chemical cross-linking of natural polymer, as reported by Thanoo et al. [16]. In this process chitosan solution (in acetic acid) was added to liquid paraffin oil containing a surfactant (span-80) resulting in formation of w/o emulsion. To this emulsion different crosslinking agents, GA (25% v/v) and TPP (3% w/w) solution was added depending upon the crosslinking density required. The microspheres formed were filtered, washed with suitable solvents and dried in vacuum desiccator.

A different concentration of chitosan solution (1.0, 1.5, 2.0 and 3% w/v) in aqueous acetic acid (3% v/v) solution was prepared and weighed amount of drug was dispersed in the chitosan solution. This dispersed phase was added to continuous phase (50 ml) consisting of light liquid paraffin containing 0.5% (w/v) span-80 as emulsifying and stabilizing agent to form water in oil (w/o) emulsion with stirring at a optimum speed of 1,200 rpm using a 3-blade propeller overhead stirrer (Remi Equipments, Mumbai, India). A measured volume of 0.5 ml of aqueous GA solution (25% v/v) was added drop wise at every 30, 60, 90 and 120 min of stirring for formulations AM-1–AM-4 and TPP (3% w/v) solution for formulations AM-5–AM-8. Stirring was continued at room temperature for 4 h to obtain microspheres, then were separated by filtration under vacuum and washed three times with petroleum ether followed by isopropyl alcohol to remove the un-entrapped drug, adhered liquid paraffin, GA and TPP. The microspheres were then finally dried in vacuum desiccator for 24 h.

The effect of various parameters such as drug to chitosan ratio, chitosan concentration and effect of different crosslinking agents (GA, 25% v/v and 3% w/w TPP) on particle size, EE and release behaviour were studied. The volume of light liquid paraffin and span-80 were kept constant. The composition of different formulations is given in Table 1.

2.4 Characterization of microspheres

2.4.1 *Percentage yield*

The yield of the chitosan microspheres was determined after complete drying from the following equation:

$$\% \text{ Yield} = \frac{\text{Dried Microspheres(mg)}}{\text{Drug (mg)} + \text{chitosan weight} + \text{cross - linker weight}}$$

2.4.2 *Determination of EE*

Encapsulation efficiency of ANS was determined by measuring the total amount of ANS present in each 20 mg sample (i.e. practical core loading) and comparing this value with the expected amount of ANS in each of the samples based on the drug loading during the preparation (i.e. theoretical core loading) [17]. Crushed microspheres (20 mg) were soaked in methanol (10 ml) for 24 h at 37°C. The solution was filtered using 0.45 μm membrane filters and after suitable dilution with mobile phase mixture, with

Table 1 Composition, percentage yield, percentage EE and percentage erosion of different formulations

Formulations	Drug (mg)	Chitosan (% w/v)	Cross-linking agent	Percentage yield	% EE	Erosion (%)
AM-1	5	1.0	GA 25% v/v	60.5 ± 3.5	30.45 ± 1.2	3.8 ± 0.5
AM-2	5	1.5	GA	68.3 ± 4.1	45.13 ± 2.5	3.6 ± 0.4
AM-3	5	2.0	GA	81.2 ± 5.2	58.26 ± 2.6	3.0 ± 0.3
AM-4	5	3.0	GA	87.6 ± 3.8	65.26 ± 3.1	2.6 ± 0.4
AM-5	5	1.0	TPP 3% w/w	58.4 ± 2.6	39.2 ± 1.5	4.1 ± 0.8
AM-6	5	1.5	TPP	71.2 ± 4.5	54.23 ± 1.9	3.8 ± 0.5
AM-7	5	2.0	TPP	79.8 ± 4.9	55.8 ± 2.1	3.4 ± 0.7
AM-8	5	3.0	TPP	83.4 ± 3.7	69.2 ± 3.2	3.1 ± 0.4

UV detector at wavelength of 210 nm. All samples were loaded in triplicate.

The percentage EE was calculated using following equation:

$$\% \text{ Encapsulation efficiency} = \frac{\text{Amount of ANS in the prepared microspheres} \times 100}{\text{Amount of drug loaded in the microspheres}}$$

2.4.3 Particle size analysis

Particle size was determined by using Malvern particle size analyzer (Malvern Instruments, UK). For Malvern particle size analysis the microspheres were first suspended in 100 ml of distilled water (containing 1% Tween-80) and subjected to sonication for 30 s and vortex mixing for 10 s before analysis.

2.4.4 Scanning electron microscopy (SEM)

The shape and surface morphology of the microspheres was examined using SEM (JSM-5610, Tokyo, Japan). An appropriate sample of microspheres was mounted on metal (aluminium) stubs, the samples were mounted onto aluminum specimen stubs using double-sided adhesive tape and fractured with a razor blade. The samples were sputter-coated with gold/palladium for 120 s at 14 mA under argon atmosphere for secondary electron emissive SEM and observed for morphology, at acceleration voltage of 15 kV at different magnifications.

2.4.5 Fourier transform infrared spectroscopy

Infrared spectra of ANS, physical mixtures—ANS + chitosan + GA, ANS + chitosan + TPP and drug loaded microspheres (AM-4 and AM-8) was recorded by using a Shimadzu FTIR 8300 spectrophotometer in the region of 4000–400 cm^{-1}. The procedure consisted of dispersing a sample (ANS, physical mixtures and drug loaded microspheres) in KBr (200–400 mg) and compressing into discs by applying a pressure of 5 tons for 5 min in a hydraulic press. The pellet was placed in the light path and the spectrum was obtained for all the samples.

2.4.6 Differential scanning calorimetry

Differential scanning calorimetry of ANS, chitosan, TPP, physical mixtures—ANS + chitosan, ANS + chitosan + TPP and drug loaded microspheres (AM-4 and AM-8) was performed by using DSC-60. The instrument comprised of calorimeter (DSC 60), flow controller (FCL 60), Thermal analyzer (TA 60) and operating software TA 60 from Shimadzu Corporation, Japan. Samples (ANS and microspheres) were placed in aluminium pans and were crimped, followed by heating under nitrogen flow (30 ml/min) at a scanning rate of 5°C/min from 25 to 250°C. Aluminium pan containing same quantity of indium was used as reference. The heat flow as a function of temperature was measured for all the samples.

2.4.7 Erosion study

The microspheres (100 mg) were immersed in phosphate buffer solution (pH 7.4) and stirred at 100 rpm for 12 h. After 12 h of immersing, microspheres were separated by centrifuge (5,000 rpm, 10 min) and dried in a temperature controlled oven at 40°C for 24 h to dry the microspheres completely, these were weighed to calculate the mass loss. Experiment was carried out in duplicate [18].

2.4.8 Swelling ratio study

The swelling ratio of blank microspheres was determined by immersion in a phosphate buffered saline (PBS pH 7.4) at room temperature for 48 h with gentle shaking. At specific time points, (0.5, 1, 2, 4, 6, 8, 12, 24 and 48 h) samples were removed and rinsed with MilliQ water. The microspheres remained intact during the process and no macroscopic pores were visible. Also the shape of microspheres remained the same. The microspheres were then blotted dry and the swollen weight (W_{SW}) was measured

and the swelling ratio (E_{SW}) was calculated according to equation as follows:

$$E_{SW} = [(W_{SW} - W_0)/W_0] \times 100$$

where E_{SW} is the swelling ratio of the microspheres, W_0 is initial dry weight of microspheres and W_{SW} is the weight of the swollen microspheres. Swelling ratio was determined using duplicate samples of microspheres [19].

2.4.9 *In vitro drug release study*

In vitro drug release of the prepared microspheres was performed using a modified dialysis sac technique [20]. Microspheres containing single dose equivalent amount of ANS were suspended in 2 ml of phosphate buffer pH 7.4, was placed in sigma dialysis sac tubing, in which sigma membrane [MWCO = Molecular weight cutoff—Mol wt. 12 KiloDaltons (KD)] used was previously soaked in phosphate buffer pH 7.4 overnight. The tube (donor compartment) containing dispersion of microspheres was then introduced into a 100 ml beaker containing 50 ml release media as phosphate buffer pH 7.4 (receptor compartment), with 0.3% Tween-80 to maintain sink condition and to improve solubility of drug, which was stirred at 600 ± 20 rpm using magnetic stirrer. Drug release was assessed by intermittently sampling the receptor media (2 ml) at predetermined time intervals (0.5, 1, 2, 4, 6, 8, 12, 24 and 48 h) and each time it was replaced with 2 ml of fresh phosphate buffer pH 7.4 [21]. The withdrawn sample was filtered through 0.45 μm membrane filter and the amount of ANS released in each sample was determined using a calibration curve by using HPLC equipped with UV detector at 210 nm. The reported values are averages of three replicates.

2.4.10 *Kinetics of drug release from microspheres*

The drug release from the optimized formulations (AM-4 and AM-8) was treated with mathematical equations such as Zero order (percent release vs t), First order kinetics (log percent release vs t), Higuchi's diffusion equation ($Q = Kt_{1/2}$), and Korsmeyer-Peppas equation ($M_t/M_\infty = Kt\ n$), where M_t is the amount of drug released at time t and M_∞ is the amount released at time infinity, thus the M_t/M_∞ is the fraction of drug released at time t, k is the kinetic constant and n is the diffusional exponent [22, 23].

2.5 Pharmacokinetic studies

The pharmacokinetic studies were carried out in male Wistar rats (150–200 g), obtained from Central Animal House, Manipal University, Manipal. The study protocol was approved by the Institutional Ethical Committee (IAEC/KMC/18/2008–2009), Kasturba Medical College, Manipal University, Manipal. They were housed and maintained at 25 ± 2°C and 50 ± 5% RH for 12 h light dark cycle. Rats were fasted and given ad libitum of water. Sodium carboxy methyl cellulose (CMC) solution, being suitable and biocompatible suspending agent used to prepare different solutions for intramuscular injection. The over night fasted animals were divided into three groups ($n = 6$) and treated intramuscularly (1.0 mg/kg) as below.

Group I: Pure ANS in 0.1% w/v sodium carboxy methyl cellulose (CMC) solution.
Group II: GA cross-linked chitosan microspheres (AM-4) in 0.1% w/v CMC solution.
Group III: TPP cross-linked chitosan microspheres (AM-8) in 0.1% w/v CMC solution.

The blood samples were collected at predetermined intervals of 0.25, 0.5, 1, 2, 4, 8, 12, 24 and 48 h post dose into heparinized tubes from the orbital sinus. The blood samples were immediately centrifuged using cold centrifugation (Remi Equipments Ltd., Mumbai, India) at 10000 rpm for 5 min and the plasma was stored at −70°C until analysis.

2.5.1 *Bioanalysis of ANS in rat plasma*

The bioanalytical method based on HPLC was used to analyze the drug in rat plasma. The method comprised of reversed phase (RP) C_{18} column (Grace Vydac®, 250 cm × 4.6 mm, 5 μm) using mobile phase, acetonitrile and 10 mM phosphate buffer pH 3.0 ± 0.2 (60:40% v/v) with a flow rate of 1 ml/min at a detection wave length of 210 nm. ANS stock solution 1 mg/ml was prepared using methanol as a diluent. Working standard solutions were prepared in the concentration range of 0.25–50 μg/ml of ANS and 10 μg/ml of valdecoxib used as an internal standard (IS). Briefly, rat plasma (90 μl) was pipetted into micro-centrifuge tubes and spiked with 10 μl of internal standard (10 μg/ml of valdecoxib–IS) and 10 μl of different standard solutions were spiked to get 0.025–5.0 μg/ml of ANS in rat plasma. To this, 200 μl of protein precipitating reagent, acetonitrile: methanol (1:1) mixture was added and vortexed for 5 min and then centrifuged at 10000 rpm for 5 min at 4°C. To the clear supernatant 100 μl of mobile phase mixture was added and injected into HPLC system. Standard curves were obtained by using drug/internal standard peak area ratio and theoretical concentration slopes, intercept and correlation coefficients were determined.

2.5.2 *Bioanalytical method validation*

The developed method was validated as per USFDA guidelines [24]. The method was evaluated for linearity using freshly prepared spiked plasma samples in the concentration range of 0.025–5.0 μg/ml. A calibration curve

for ANS was constructed prior to the experiments and the acceptance criteria for the calibration curve was with a correlation (R^2) value of 0.995 or more. The intra-batch accuracy and precision were determined by analysis of six replicates each of low, medium and high concentration quality control samples; while inter-batch accuracy and precision were determined by the analysis of these quality control samples on three separate occasions. The quality control samples were prepared at concentrations of 0.025 μg/ml (low), 0.5 μg/ml (medium) and 5.0 μg/ml (high) for ANS. The overall precision of the method was expressed as relative standard deviation (RSD) and accuracy of the method expressed as percent to true value.

2.5.3 *Recovery of rat plasma*

The stock solution of ANS was added to rat plasma to yield final concentrations of 0.025, 0.5 and 5.0 μg/ml. Further, ANS stock solution was diluted using methanol as diluent to obtain concentrations of 0.025, 0.5 and 5.0 μg/ml. Both plasma samples and the diluted solutions were processed as described earlier and the ratio of peak area (plasma sample/diluted solution) for ANS was used to calculate the recovery in rat plasma.

2.6 In vitro–in vivo correlation (IVIVC)

In the present study IVIVC was carried out using Level A correlation [25] and the amount of drug absorbed is calculated by deconvolution technique using Wagner Nelson method. The in vivo absorption profile is plotted against the in vitro dissolution profile to obtain a correlation. If the relationship becomes linear with a slope of 1, then the curves are superimposable, and there is a 1:1 relationship, which defines point to point correlation [26].

2.7 Statistical analysis

The pharmacokinetic parameters were calculated by using PK Solutions® 2 software, Montrose, CO, USA. One way ANOVA, with Tukey posthoc test was used to analyze the data using Graphpad Prism® version 4.03 and $p < 0.05$ was considered statistically significant.

3 Results

3.1 Development of ANS analytical method

In the present study a new analytical HPLC method was developed for the estimation of ANS in developed multiparticulate formulations. The retention time (RT) of ANS was found to be 7.2 ± 0.2 min and the correlation coefficient was found to be ≥ 0.999 indicating method linearity [27]. The relative standard deviation of inter-day and intra-day was found to < 1 at all levels. The overall recovery was found to be 97.5–101.5% indicating accuracy of the method.

3.2 Percentage yield

The percentage practical yield of all formulations is shown in Table 1. Practical yield of formulations (AM-01–AM-04) using GA as cross-linking agent was found to be in the range of 60.5 ± 3.5–87.6 ± 3.8% and for formulations (AM-05–AM-08) using TPP as cross-linking agent was in the range of 58.4 ± 2.6 and 83.4 ± 3.7%.

3.3 Encapsulation efficiency

The EE of different formulations prepared at 1200 rpm varied from 30.45 ± 1.2 to 69.25 ± 4.2%. Different batches were prepared by varying the chitosan concentration. The results are tabulated in Table 1. With 3% w/v chitosan, %EE was increased up to 65.26 ± 3.7 for AM-4 and 69.25 ± 4.2 for AM-8 formulations. The increase in GA and TPP (50% v/v and 4% w/w) concentration, %EE was found to be 66.54 ± 2.4 and 71.03 ± 2.9. The microspheres prepared with high concentration of chitosan (> 3% w/v) resulted in decreased %EE, 63.68 ± 1.8 for 25% v/v GA and 67.92 ± 2.5 with 3% w/w TPP with slight aggregation of the particles.

3.4 Particle size analysis and surface morphology

The mean particle size distribution of different formulations is depicted in Fig. 1a. The particle size of all formulations was found to be in the range of 72.5 ± 0.5–157.98 ± 1.5 μm. The results also indicated no significant variation in mean particle size of AM-4 (150.92 ± 0.5 μm) and AM-8 (157.98 ± 1.5 μm) as shown in Fig. 1b and c, the microspheres prepared using different crosslinking agents at optimum speed of 1,200 rpm. SEM photographs of formulations AM-4 and AM-8 are shown in Fig. 2a and b.

3.5 FTIR studies

The FTIR results of ANS, physical mixtures—ANS + chitosan + GA, ANS + chitosan + TPP and drug loaded microspheres are depicted in Table 2. FTIR spectra of drug demonstrates the characteristic absorption bands (major peaks) at wave numbers, 3099.7 cm^{-1} due to aromatic C–H stretch, 2982.05 and 2918.4 cm^{-1} due to CH_3 aliphatic stretch, 2234.64 cm^{-1} (medium C≡N stretch), 1604.83 cm^{-1} (skeletal vibrations) due to C=C of aromatic ring, 1572.04 and 1502.6 cm^{-1} due to aromatic ring, 1537.32 cm^{-1} due to C=N stretching, 1465.95, 1357.93 and 1390.72 cm^{-1} due to gem dimethyl groups, 1273.06

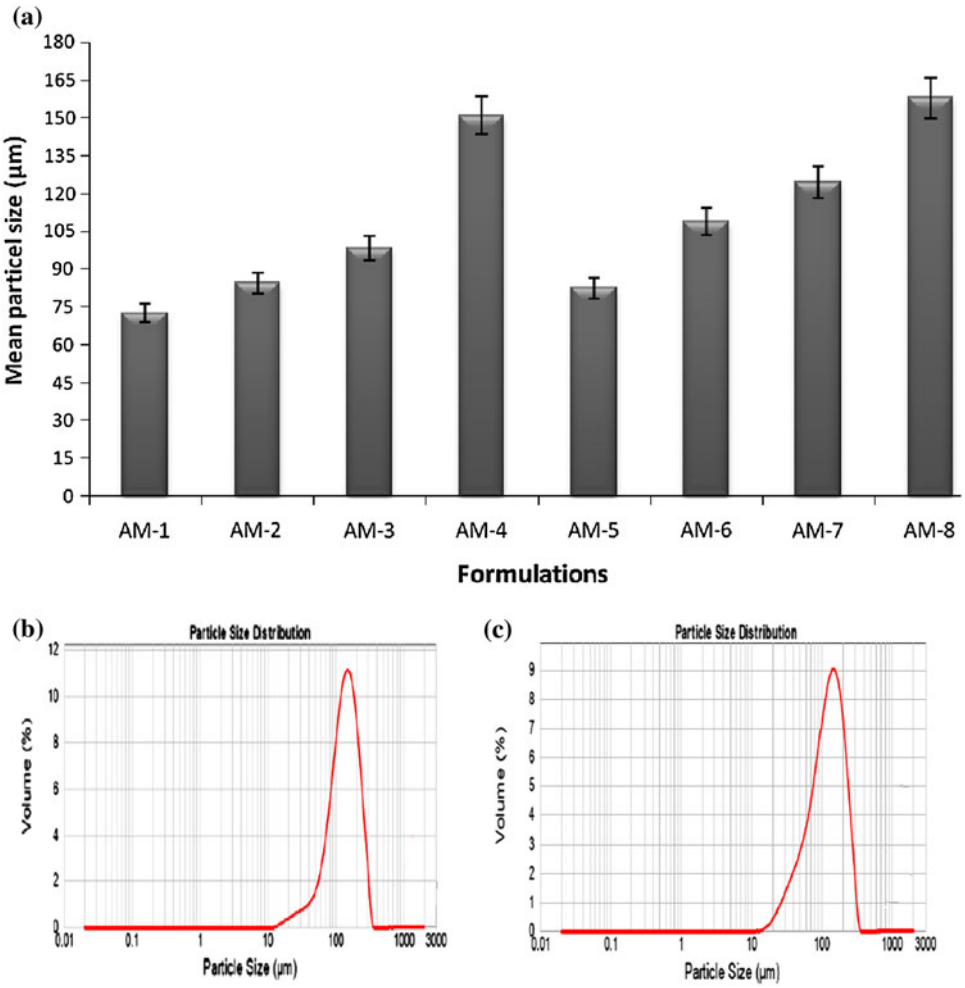

Fig. 1 Mean particle size distribution of anastrozole-loaded chitosan microspheres. (**a**) The mean particle size distribution of different formulations (AM-1 to AM-8) (**b**) AM-4 and (**c**) AM-8

and 1201.69 cm^{-1} due to tertiary butyl type skeletal vibration, and 761.91 cm^{-1} due to out of plane C–H bend (aromatic ring), respectively [28].

3.6 DSC analysis

The DSC thermograms of different samples are depicted in Fig. 3. The DSC thermogram of ANS exhibited a sharp endothermic peak at 83.87°C, corresponding to its melting point [28, 29]. The physical mixture of ANS and polymer showed nearly the same thermal behaviour indicating there was no interaction between the drug and polymer in solid state. The peak of the drug did not appear in the thermogram of any type of the prepared microspheres.

3.7 Erosion of matrices

These results of erosion studies are given in Table 1. Among the cross-linking agents used, GA-crosslinked microspheres (AM-1–AM-4) showed the least erosion, 2.6 ± 0.4–3.8 ± 0.5%, where as TPP-cross linked microspheres AM-5–AM-8, showed erosion of 3.1 ± 0.4–4.1 ± 0.8% respectively.

3.8 Swelling ratio

The swelling study experiment results of GA–chitosan and TPP–chitosan microspheres are depicted in Fig. 4. The swelling capacity (%) chitosan microspheres cross-linked with different cross-linking agents (GA, AM-4 and TPP, AM-8) increased with time.

3.9 In vitro drug release study

The results of in vitro release were depicted in Fig. 5a and b. The formulations AM-1, AM-2, AM-5 and AM-6 showed more than 95 ± 2.2% drug release in 48 h, formulations AM-3 and AM-7 showed more than 80 ± 1.4% in 48 h, whereas AM-4 and AM-8 showed 69.45 ± 1.2 and 73.04 ± 1.6% in 48 h respectively (supported by swelling behaviour

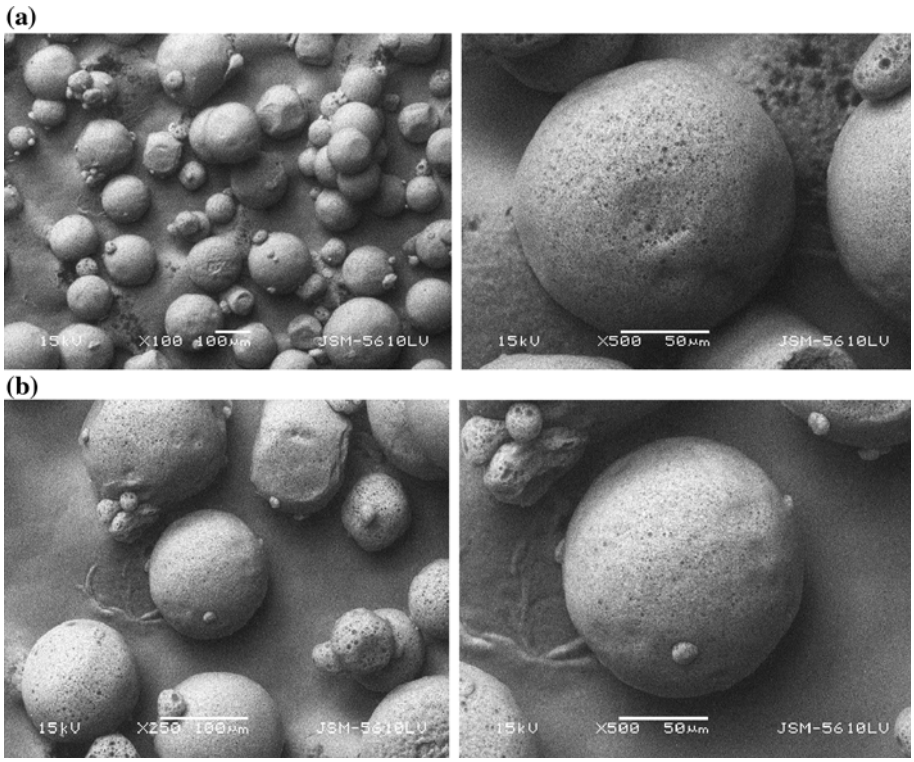

Fig. 2 **a** Shape and surface morphology of formulation AM-4 and **b** Shape and surface morphology of formulation AM-8

Table 2 FTIR spectra of (a) ANS; (b) physical mixtures—ANS + chitosan + GA, (c) ANS + chitosan + TPP and drug loaded microspheres, (d) AM-4 and (e) AM-8

Sample	Composition	Major peaks (wave numbers, cm^{-1})
a	Pure ANS	3099.71, 2982.05, 2918.4, 2330.09, 2234.64, 1604.83, 1572.04, 1537.32, 1502.6, 1465.95, 1357.93, 1390.72, 1273.06, 1201.69 and 761.91
b	ANS + chitosan + glutaraldehyde	2959.02, 2333.12, 1605.59, 1600.01, 1504.31, 1416.76, 1355.04, 1215.19 and 733.94
c	ANS + chitosan + TPP	3103.57, 2355.16, 2328.16, 1608.69, 1515.21, 1475.69, 1375.29, 1276.92, 1202.34 and 775.82
d	AM-04	2923.22, 2357.09, 2329.12, 1659.80, 1531.53, 1221.95, 1392.65 and 892.11
e	AM-08	2920.32, 2355.16, 2328.16, 1734.06, 1633.76, 1375.39, 1062.81, 920.08 and 879.57

studies). Higher the swelling capacity of the matrix, higher the amount of drug diffused out from the matrix.

Based on physicochemical parameters, formulations AM-4 and AM-8 were selected as optimized batches, between these formulations the calculated f1 value is 31.31 and f2 value is 50.67. From these values it can be revealed that there is a considerable difference in the drug release profiles between the two formulations [30]. Further, these two formulations were selected for pharmacokinetics study in rats.

3.10 Release kinetics

From the release kinetics data, based on the highest coefficient of determination value (R^2), which is nearing to unity, microspheres were found to follow Higuchi-matrix kinetics (Table 3). When plotted in accordance with Korsemeyer–Peppas method, the n-value $0.5 < n < 1.0$ for AM-4 and AM-8 microspheres suggest the anomalous (non-Fickian) transport. Percentage drug release for AM-4 and AM-8 found to be 69.45 ± 1.2 and 73.04 ± 1.6% in 48 h respectively and is depicted as sustained release when compared to all the other formulations.

3.11 Bioanalytical method validation

3.11.1 Linearity

The peak area ratio of ANS to IS in rat plasma was linear with respect to the analyte concentration over the range of 0.025–5.0 µg/ml. The correlation coefficient (R^2) of ANS was greater than 0.995 for the concentrations used. The LLOQ was established as 0.025 µg/ml, the lowest concentration on the calibration curve that could quantify ANS with acceptable precision and accuracy.

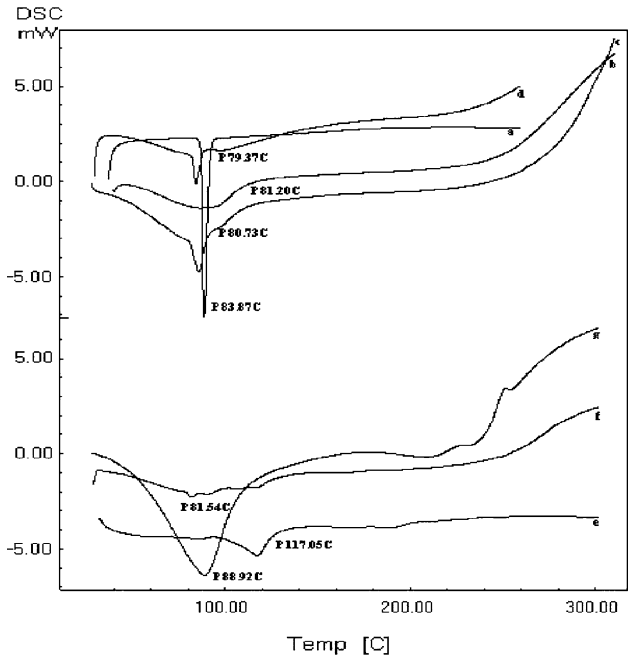

Fig. 3 DSC thermograms of **a** ANS; **b** chitosan; **c** TPP; **d** physical mixtures—ANS + chitosan + GA; **e** ANS + chitosan + TPP and drug loaded microspheres; **f** AM-4; **g** AM-8

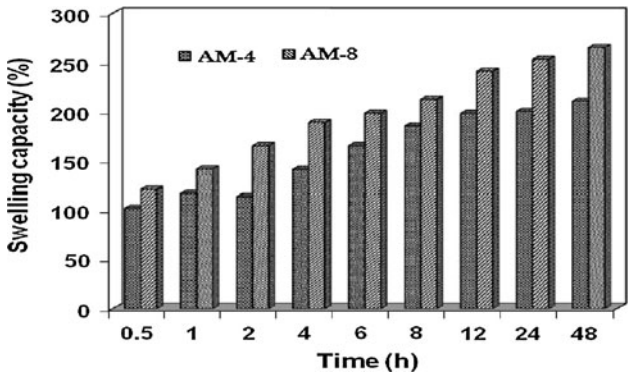

Fig. 4 Swelling capacity studies of formulations AM-4 and AM-8

3.11.2 Precision and accuracy

The results shown in Table 4 indicate that this method is reproducible for replicate analysis of ANS in rat plasma. The mean precision defined by RSD, ranged from 4.45 to 11.16% whereas the mean accuracy ranged between 88.40 and 98.93%.

3.11.3 Recovery

The use of acetonitrile: methanol (1:1) as extracting solvent was selected for protein precipitation method and resulted in about 65–70% recovery of ANS in rat plasma.

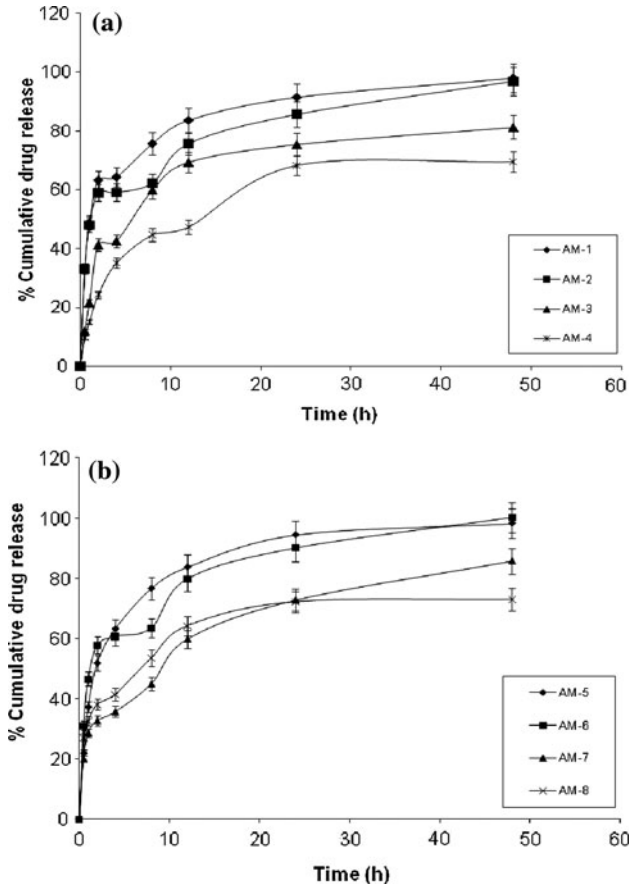

Fig. 5 **a**, **b** In vitro release profile of chitosan microspheres (AM-1–AM-8)

Table 3 Kinetic values obtained from mathematical model treatment with best fit models

Microspheres	AM-04	AM-08
Zero order (R^2)	0.629	0.567
First order (R^2)	0.7335	0.8494
Higuchi model (R^2)	0.9972	0.9951
Korsemeyer–Peppas (n)	0.7516	0.5713
Best fit model	Higuchi	Higuchi

3.12 Pharmacokinetics

The plasma concentration time profile of pure drug, GA crosslinked (AM-4) and TPP cross-linked (AM-8) chitosan microspheres are depicted in Fig. 6. There was significant difference in the pharmacokinetic parameters when ANS was formulated in the form of microspheres (AM-4 and AM-8). The results showed that area under concentration–time curve ($AUC_{(0-\infty)}$) value of AM-4 and AM-8 formulations was found to be 50.8 ± 5.39 and 55.2 ± 6.48 μg h/ml compared to pure drug, showed $AUC_{(0-\infty)}$ value of 16.0 ± 1.35 μg h/ml respectively. Chitosan microspheres (AM-4

Table 4 Results of precision and accuracy of ANS in rat plasma

Initial concentration (μg/ml)	Obtained concentration (mean ± SD, μg/ml)	Precision (%)	Accuracy (%)
Intra-batch ($n = 6$)			
0.025	0.022 ± 0.004	9.2	88.40
0.5	0.45 ± 0.05	5.92	95.12
5.0	4.75 ± 0.41	4.88	97.65
Inter-batch ($n = 3$)			
0.025	0.024 ± 0.009	11.16	92.48
0.5	0.48 ± 0.08	4.38	98.93
5.0	4.88 ± 0.52	4.45	95.78

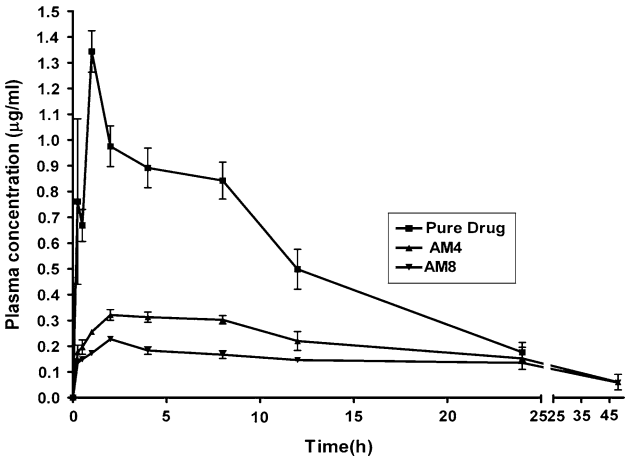

Fig. 6 Plasma concentration versus time profile for pure drug, AM-4 and AM-8

and AM-8) formulated had significantly improved the systemic exposure, reduced the clearance (Cl) and raised the volume of distribution (Vd) as shown in Table 5. The mean residence time (MRT) of AM-04 and AM-08 formulations was found to be 26.6 ± 1.5 and 36.2 ± 2.5 h compared to pure drug having a MRT of 11.4 ± 1.25 h respectively. Also, elimination rate constant (Kel) was found to be slow, 0.062 ± 0.02 and 0.0433 ± 0.01 h^{-1} with elimination half life ($t_{1/2}$) of 19.1 ± 2.36 and 25.9 ± 4.45 h for formulations AM-4 and AM-8, whereas pure drug showed a Kel of 0.296 ± 0.12 h^{-1} with low elimination half life of 7.4 ± 0.15 h respectively.

4 Discussion

The objective of the present study was to develop a formulation of ANS microspheres by w/o emulsification crosslinking method using chitosan as sustained release biodegradable polymer. In sustained release technology, biodegradable polymeric carriers like chitosan offer

Table 5 Results of pharmacokinetic parameters for pure drug, AM-4 and AM-8

Parameters	Mean ± SD		
	Pure drug	AM-4	AM-8
$AUC_{(0-\infty)}$ (μg h/ml)	16.0 ± 1.35	50.8 ± 5.39*	55.2 ± 6.48*
C_{max} (μg/ml)	1.3 ± 2.56	0.3 ± 0.08*	0.2 ± 0.06*
MRT (h)	11.4 ± 1.25	26.6 ± 1.5*	36.2 ± 2.5*
T_{max} (h)	1.0	2.0*	2.0*
Cl (l/h)	0.662 ± 0.015	0.101 ± 0.01*	0.121 ± 0.012*
Vd (l/kg)	0.668 ± 0.01	2.79 ± 0.31*	4.5 ± 0.46*
Kel (h^{-1})	0.296 ± 0.12	0.062 ± 0.02*	0.0433 ± 0.01*
$t_{1/2}$ (h)	7.4 ± 0.15	19.1 ± 2.36*	25.9 ± 4.45*

$P < 0.05$ at 95% CI (Tukey posthoc test); * Significantly different compared to pure drug

C_{max} maximum plasma concentration; T_{max} time to reach maximum plasma concentration; $AUC_{0-\infty}$ area under the curve; *MRT* mean residence time; *Cl* clearance; *Vd* volume of distribution; *Kel* elimination rate constant; $t_{1/2}$ half-life time. The data represents mean ± SD ($n = 6$)

potential advantages for the prolonged release of low molecular weight compounds. In the earlier studies molecules such as bovine serum albumin, diphtheria toxoid and bisphosphonates have been successfully incorporated into chitosan microspheres [31]. Matrices made of hydrophilic polymers such as chitosan modify the release of a loaded drug with the help of crosslinking agents.

ANS is a new-generation potent nonsteroidal aromatase inhibitor of the triazole class. Previously, analytical methods have been reported for the estimation of the drug using HPLC-MS, Gas chromatography mass spectrum (GCMS), etc. [28, 32, 33]. Since, the present study was focused on development of the multiparticulate carrier system for the delivery of drug, estimation of drug in the developed formulation requires highly specific analytical method to avoid the interference from the matrix. Hence HPLC method was developed and validated for the estimation of ANS. The specificity of the method was been evaluated by processing the blank microsphere unlike sample processing and injected into the HPLC system. Any interference at the drug retention time was monitored by sequential injection of standard ANS. The developed method was found to be specific as there was no interference at the retention time of the analyte.

Optimization of various formulation parameters in preparation of chitosan microspheres were carried out initially. The light liquid paraffin containing 0.5% span-80 (stabilizing agent) as external phase with a stirring speed of 1,200 rpm was found to be optimum for maximum yield of chitosan microspheres. The stirring speed was optimized by preparing blank chitosan microspheres at different

speeds. Initially the microspheres were prepared with low speed of 500 rpm, then with 1,000–1,200 rpm, followed by 1,500 rpm and above. Based on the % practical yield, mean particle size distribution and surface morphology studies of blank microspheres an optimum speed of 1,200 rpm was used for preparation of drug loaded microspheres.

It was observed that non-cross-linked chitosan microspheres cannot be kept suspended in water because of swelling and dissolution. As a result, the release rate of the drug from non-crosslinked chitosan microspheres was rapid. Therefore, cross-linked chitosan microspheres are suitable for the sustained release application [34]. The cross-linkers selected for preparation of chitosan microspheres were covalently cross-linking agent, GA 25% aqueous solution and ionic cross-linking agent, TPP 3% w/w. TPP has also been demonstrated as a stabilizing agent for the preparation of chitosan microspheres [35]. During the process when cross-linker was added at once, due to the anionic structure of TPP, it reacts with polymer and similarly, GA covalently binds with polymer, produced bulky mass (jelly like structures) or lump. To avoid this they were added slowly for efficient crosslinking.

Further drug to polymer ratio was varied to see the effect on microspheres formation. As polymer ratio in the formulation (AM-1–AM-8) increased, the product yield was also found to be increased. The low percentage yield in some formulation may be due to loss during washing process. Among all the formulations studied AM-4 and AM-8 showed maximum practical yield followed by other formulations. The EE was found to be affected by chitosan concentration and was increased progressively with increase in chitosan concentration. This may be attributed to the greater availability of active binding sites in the polymeric chains for drug molecules. Also an increased concentration of chitosan resulted in the formation of larger microspheres. The incorporation efficiency was higher for AM-4 and AM-8 respectively. Further increase in chitosan concentration (> 3), there was no significant difference in the EE. It could be due to saturation of the polymer in the formulation with formation of bulky mass. Since highly concentrated solutions made the addition procedure difficult, therefore the optimum concentration of chitosan was chosen as 3.0% w/v.

Increase in the concentration of cross-linkers resulted in hardening of microspheres, difficult to suspend in aqueous media and increase in concentration of cross-linking agents had no significant effect on the EE [36]. At lower concentration microspheres were not formed which may be due to insufficient cross-linking.

Particle size analysis results indicated proportional increase in the mean particle size of the microspheres with increase in amount of chitosan concentration in all formulations. The speed of rotation was found to influence the particle size. It was observed that when the speed of stirrer was at 500 rpm the microspheres obtained were bigger in size and irregular in shape. There was no formation of spherical microspheres. This could be due to inadequate agitation to disperse the inner phase in the total mass. Therefore, particles were found to settle at the bottom of vessel. The desired mean particle size range (100–150 μm) and spherical microspheres were obtained at optimized stirring speed of 1,200 rpm. When the speed was increased up to 2000 rpm microspheres of smaller particle size (50 μm) were obtained [37]. However, at higher stirring speeds, high turbulence was formed, caused frothing and adhesion to the container wall. There was increase in particle size with increase in polymer concentration, probably due to increasing viscosity of the dispersed phase and higher resistance against shear forces during emulsification. Morphology of the chitosan microspheres prepared was found to be discrete, well formed, smooth, slightly porous and spherical in shape. The microspheres formulated were aligned in intimate contact with spherical like structures. All the particles were discrete entities, did not cause aggregation of particles after drying. These particles were readily redispersible. This indicates that drug molecules are completely dispersed in the polymeric structures lead to formation of microspheres.

The stability of ANS in the chitosan microspheres was investigated by FTIR. Results indicated that the wave numbers present in the IR spectra of ANS were also found in the physical mixtures with comparable intensities, attributing to the compatibility of drug–polymer. Almost identical absorption bands were obtained from physical mixtures and drug loaded chitosan microspheres, but with lower intensity. FTIR analysis indicated that there are no chemical interactions in the solid state between drug and the polymer, indicating stable nature of ANS in the freshly prepared chitosan microspheres.

Further DSC analysis was carried out as a useful tool in the investigation of solid-state interactions. The main interactions in preparation of chitosan microspheres forming the network are covalent or ionic bonds depending on the nature of crosslinkers. In the case of chemical crosslinking, the aldehyde groups form covalent imine bonds with amino groups of chitosan, due to resonance established with adjacent double ethylenic bonds via Schiffs reaction. Ionic interactions between negative charges of crosslinker (TPP) and positively charged groups of chitosan are the main interactions inside network. The absence of peak of the drug in the thermogram of prepared microspheres clearly indicated that drug existed in an amorphous and uniformly dispersed form at the molecular level in the microspheres.

Erosion study is an important parameter to see the effect of cross-linking. The percentage erosion of TPP-crosslinked

chitosan microspheres was higher than GA-crosslinked chitosan microspheres because of actual chemical reaction between GA and chitosan is a stronger and more rigid matrix than ionically crosslinked matrix TPP [21, 38]. Erosion of polymer was slow when the concentration of chitosan was increased from 1.0 to 3.0% w/v in the formulations.

Also the swelling capacity of the cross-linked chitosan microspheres was influenced by the nature of the cross-linking, i.e. chemical or ionic cross-linking. Chitosan microspheres cross-linked with GA (chemical cross-linking) exhibited lower swelling capacity when compared to crosslinked with TPP (ionic cross-linking). Based on the nature of the cross-linkers, chemical or ionic cross-linking agents, the main interactions forming the network are covalent or ionic bonds [39]. In case of chemical cross-linking, the aldehyde groups form covalent imine bonds with the amino groups of chitosan, due to resonance established with adjacent double ethylenic bonds. These aldehydes allow direct reaction in aqueous media, under mild conditions with or without the addition of auxiliary molecules such as reducers. Ionic interactions between the negative charges of the cross-linker (TPP) and positively charged groups of chitosan are the main interactions inside. Hence GA-chitosan microspheres are more rigid matrices exhibited lower swelling capacity when compared to TPP-chitosan microspheres. There was no significant difference in percentage erosion and swelling capacity by increasing chitosan concentration above 3.0% w/v.

In vitro drug release studies of chitosan microspheres (AM-1–AM-8) showed an initial rapid release followed by sustained release, (diffusion processes) one occurring after the other. The diffusion of aqueous media into the matrix followed by drug diffusion through pores into the diffusion media. An initial rapid release was because of non encapsulated drug from the surface of microspheres, followed by a slow release rate controlled by the rate of swelling of the polymeric matrix and diffusion of drug from the rigidity of crosslinked microspheres [12]. The rate of ANS release reduced with the increasing concentration of chitosan, (drug quantity kept constant—5 mg) due to high efficiency of drug entrapped in polymer matrix. About 25% drug was released in the initial hour for all formulations.

Differences in drug release between optimized formulations were confirmed by model independent approach using a similarity factor as per USFDA guidelines. As per the guidelines, for curves to be considered similar, f1 values should be close to 0, and f2 values should be close to 100 [30]. Application of model independent approach on AM-4 and AM-8 batches revealed that there is a considerable difference in the drug release profile between the two formulations.

In vitro drug release data obtained were grouped into mathematical models of data treatment in order to analyze the mechanism involved in drug release from microspheres. Drug release kinetics is an important evaluation parameter in pharmaceutical dosage forms. The knowledge of the mechanism and kinetics of drug release from these microspheres indicate its performance and gives proof for adequateness of its design. Drug release from microspheres involves mass transfer phenomenon involving diffusion of drug from higher concentration to low concentration region in surrounding liquid. To accomplish these studies, cumulative profiles of the dissolved drug are described using mathematical equations/models. These models illustrate the drug release from pharmaceutical systems when it results from a simple phenomenon or when that phenomenon, by the fact of being the rate-limiting step, conditions all other processes. In the present study, the best describing kinetic models used are Zero order, First order, Higuchi model and Korsmeyer–Peppas model [22]. The Higuchi and Zero order models represent two phenomena, that is, transport of the drug from the system and drug release. The Korsmeyer–Peppas model illustrates the decision parameter between these two. The criteria to choose best fit model was based on the coefficient of determination, R^2, to assess the best fit of a model equation. It is assumed from release kinetics that diffusion of the drug is a dominant mechanism throughout the release period. The embedding bioerodable polymers used in the formulation helps in diffusion of the drug from the matrix because of concentration gradient. Hence changing the concentration of polymer material, both the dissolution rate and kinetic profile can be controlled. It means that water diffusion and also the polymer relaxation have an essential role in the drug release [40].

Pharmacokinetics study was carried out for the optimized formulations. The bioanalytical method used was sensitive, accurate and precise to quantify the ANS in rat plasma. The plasma drug concentration for AM-4 and AM-8 was detectable up to 48 h which may be due to the slow Cl rate leading to drastic increase in elimination half life [41]. The area under concentration–time curve $AUC_{(0-\infty)}$, for AM-4 and AM-8 was 2–3 fold times higher values compared to pure drug, on the other hand, C_{max} for microspheres was 3–4 times less compared to pure drug due to maximum encapsulation of drug in the polymer. The t_{max}, for pure drug and microspheres (AM-4 and AM-8) was found to be 1 and 2 h respectively. This may be attributed to the sustained release of ANS from chitosan microspheres up to 48 h, which correlates well with the in vitro release data. The in vitro release profile had good correlation to the in vivo results by measuring plasma drug concentration profile in pharmacokinetics experiment after intramuscular injection to rats with R^2 value of 0.8564 and 0.908 for AM-4 and

AM-8 formulations respectively. It indicates that prolonged release of microspheres had markedly decreased the elimination rate constant. The reasons for long sustained release were considered to be the small volume of tissue fluid and slow rate of blood stream in muscle as well as the low solubility of ANS in release medium, resulting in the low release rate in vitro and in vivo. The encapsulated drug in the microspheres facilitated for drug to release in sustained release fashion. Therefore, ANS loaded microspheres were formulated successfully suitable for intramuscular administration to improve its pharmacokinetic parameters.

In vitro–in vivo correlation study was carried out which describe a relationship between the in vitro dissolution/release versus the in vivo absorption. Generally the in vitro property is the rate or extent of drug dissolution or release while an in vivo response is the plasma drug concentration or amount of drug absorbed. Correlation levels are classified as Level A, Level B, Level C and Level D correlations. Since Level A correlation is most informative and is recommended by Center for Drug Evaluation and Research (CDER), USFDA [25]. In the present study IVIVC was carried out using Level A correlation. For development of IVIVC it is required to know the cumulative amount absorbed or the percentage absorbed which can be calculated using Wagner Nelson method, Loo–Riegelman method or model independent deconvolution method. Amount of drug absorbed is calculated by deconvolution technique—Wagner Nelson method. The in vitro and in vivo data showed that the release of ANS in the chitosan microspheres was slow and lasted for a long time. There was no statistically significant difference among both the formulations. But both showed statistically significant changes ($P < 0.05$ at 95% CI) in pharmacokinetics profile compared to pure drug.

5 Conclusion

Anastrozole microspheres were prepared successfully by using modified single emulsion technique. The highest encapsulation of ANS within chitosan microspheres could be obtained by optimizing the formulation variables. The assessment of release kinetics showed that drug release followed Higuchi-matrix model. A sustained delivery system of ANS was formulated to provide anticancer therapy with biodegradable carrier system. Thus results demonstrated that formulated microspheres could be an alternative drug delivery through parenteral route for the long-term treatment of breast cancer which is safe and cost effective for better therapeutic efficacy with reduced toxicity.

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Conflict of interest None.

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