Controlled release alginate-chitosan microspheres of tolmetin sodium prepared by internal gelation technique and characterized by response surface modeling

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Tolmetin sodium (TS) is a powerful non-steroidal mitigating drug for the treatment of rheumatoid joint inflammation, osteoarthritis, and adolescent rheumatoid joint pain. In addition to its gastrointestinal (GIT) problems, TS has a short biological half-life (1 hr). In a trial to overcome these side effects and control the rate of (TS) release, chitosan coated alginate microspheres are recommended. A Box-Behnken experimental design was employed to produce controlled release microspheres of TS in the sodium alginate and chitosan copolymers (Alg-Ch) by emulsification internal gelation methodology. The effect of critical formulation variables namely, drug to polymer ratio (D:P ratio), speed of rotation and span 80% on drug encapsulation efficiency (% EE), drug release at the end of 2 hours (Rel_2) and drug release at the end of 8 hours (Rel_8) were analyzed using response surface modeling. The parameters were assessed using the F test and mathematical models containing only the significant terms were generated for each parameter using multiple linear regression analysis. The produced microspheres were spherical in shape with extensive pores at D:P ratio 1:1 and small pores at a drug to polymer ratio (D:P ratio) 1:3. Differential scanning calorimetry (DSC) affirmed the steady character of TS in microspheres and revealed their crystalline form. All formulation variables examined exerted a significant influence on the drug release, whereas the speed emerged as a lone factor significantly influencing % EE. Increasing the D: P ratio decreases the release of the drug after two and 8 hours. The increase in speed results in an increase in drug release after two and eight hours. The drug release from the microspheres followed zero order kinetics. TS Alg-Ch microspheres exhibited a significant anti-inflammatory effect on incited rat paw edema after eight hours. These results revealed that the internal gelation technique is a promising method to control TS release and eradicate GIT side effects using Alg-Ch copolymers.

Keywords: Tolmetin Sodium. Controlled Release. Alginate. Chitosan. Response Surface.

INTRODUCTION

TS is an effective anti-inflammatory and pain-relieving drug in clinical practice and it represses both isoforms of cyclo-oxygenase enzyme. The inhibition of cyclo-oxygenase enzyme COX–I result in a number of undesirable effects such as GIT complaints.

Moreover, TS has a short plasma half-life (about 1–2 hours) that necessitates frequent administration of the drug through the daytime (Jelvehgari *et al.*, 2010). Therefore, TS is highly recommended being prescribed in a controlled release dosage form. Furthermore, it would contribute to hold encapsulated TS during emulsification, decrease its loss during microspheres recovery (Palmieri *et al.*, 2002), and may provide a controlled release effect. Consequently, the aim of this work was to control TS release using oral dosage formulations.

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In this paper, a novel method of emulsification/ internal gelation has been recommended as another option to ionic gelation in the microencapsulation of many drugs (Ahmed *et al.*, 2013) including sensitive biologicals, for example, protein drugs (Chan, Lee, Heng, 2002). Alginate microspheres with small particle size and high % EE can be produced by this methodology (Chan, Lee, Heng, 2002). The trouble in utilizing dispersion/external gelation systems with ionic polysaccharide is that the calcium source (CaCl$_2$) is insoluble in the oil phase. As an option, internal gelation of the scattered alginate might be started by discharging Ca^{2+} from an insoluble complex (calcium salt) through pH diminishment (Rajinikanth, Sankar, Mishra, 2003, Zou *et al.*, 2011, Ahmed *et al.*, 2013). Alginates, which are normally founded in brown seaweed and algae have gotten much consideration in pharmaceutical dosage forms, especially as a controlled drug delivery vehicle (Chen, Subirade, 2007). In this context, the use of alginate microspheres as an oral delivery system for TS seems very attractive. The alginate network could shield the medication from antagonistic conditions.

Alginate has mucoadhesive properties, which could expand the contact time amongst microspheres and absorptive sites, and in this way could enhance the uptake of the encapsulated drug. The low harmfulness and low immunogenicity of alginate make this polymer a safe network (Silva *et al.*, 2006, Paredes Juárez *et al.*, 2014). Much attention has been given in recent years to the use of Alg-Ch polyelectrolyte complex in controlled drug delivery.

Chitosan and its subordinates generate particular interest as a biodegradable polymer used in controlling drug release. It is a natural biopolymer consisting of β-1 → 4 linked 2-amino-2-deoxy-glucopyranose (GlcN) and 2-acetamido-2-deoxy-β-d-glucopyranose (GlcNAc) residues, fabricated industrially on a substantial scale by antacid *N*-deacetylation of chitin, an inexhaustible biopolymer separated from the exoskeleton of shellfish, for example, crabs and shrimps (Thaya *et al.*, 2018). Chitosan-alginate microspheres have been widely used to encapsulate drugs (Thaya *et al.*, 2018). The use of chitosan has been reported for coating alginate microspheres to alter the diffusion rate of encapsulated substances through the interpolymeric chitosan-alginate matrix (Motwani *et al.*, 2008). The prepared microspheres were assessed for their morphology and surface structure, average particle size, production yield, drug loading efficiency, and drug release characteristics.

MATERIAL AND METHODS

Material

TS was kindly provided by Minapharm Co. for pharmaceuticals (10th Ramadan, Cairo, Egypt). Sodium alginate (NaA), Low molecular weight (LMW) chitosan: Sigma Chemical Co., St. Louis (USA). Calcium carbonate (CaCO$_3$), methylene chloride, glacial acetic acid, light liquid paraffin and potassium dihydrogen orthophosphate, Adwia, El-Nasr Pharm. Chem. Co. (Egypt). Other materials and solvents are of reagent or analytical grade, and they were used without further purification.

Experimental Design

A Box-Behnken experimental design was utilized to investigate and optimize the formulation parameters of TS microspheres preparation for maximum % EE and controlled drug delivery after two and 8 hours (Yetilmezsoy, Demirel, Vanderbei, 2009). This design is used as it requires fewer treatment combinations than other designs in cases involving more than two dependent variables (El-Shenawy *et al.*, 2017). The Box- Behnken design is also ratable and contains statistical "missing corners" which may be useful when the experimenter is trying to avoid combined factor extremes. This property prevents a potential loss of data in those cases (Motwani *et al.*, 2008). A 3 factor 3 levels design was employed to design controlled release microspheres of TS. The three independent formulation variables analyzed during the study were D:P ratio(X_1), revolution per minute (X_2) and span 80% (X_3). The selected factors with the actual and coded levels as per the design are represented in Table I. According to this design, 15 formulae of TS microspheres were prepared. Three levels of the speed were used 200, 400 and 600 rpm denoted the values -1, 0 and +1 in the above design, respectively. Drug: polymer ratio was varied to be (1:1), (1:2) and (1:3), also denoted the values -1, 0 and +1, respectively. Lastly, span 80% was chosen to be 1%, 1.5% and 2% denoted -1, 0 and +1 value, respectively. The dependent variables to be tested for the prepared TS microspheres were chosen to be the loading efficiency (Y_1), release at the end of 2 h (Y_2) and release at the end of 8 h (Y_3).

TABLE I - Composition of different formulae of TS-Alg-Ch microspheres

Formula No.	TS (g)	Ch. (g)	Na-Alg. (g)	$CaCo_3$ (g)	Span 80 (mL)	Speed	Total weight (g)
F1	0.5	0.25	0.25	0.375	1.5	200	1.375
F2	0.5	0.25	0.25	0.375	1.0	400	1.375
F3	0.5	0.25	0.25	0.375	2	400	1.375
F4	0.5	0.25	0.25	0.375	1.5	600	1.375
F5	0.5	0.75	0.25	0.375	1.0	200	1.875
F6	0.5	0.75	0.25	0.375	2	200	1.875
F7	0.5	0.75	0.25	0.375	1.5	400	1.875
F8	0.5	0.75	0.25	0.375	1.5	400	1.875
F9	0.5	0.75	0.25	0.375	1.5	400	1.875
F10	0.5	0.75	0.25	0.375	1.0	600	1.875
F11	0.5	0.75	0.25	0.375	2	600	1.875
F12	0.5	1.25	0.25	0.375	1.5	200	2.375
F13	0.5	1.25	0.25	0.375	1.0	400	2.375
F14	0.5	1.25	0.25	0.375	2	400	2.375
F15	0.5	1.25	0.25	0.375	1.5	600	2.375

Preparation of Alginate Coated Microspheres

Composition of different suggested formulae of TS microspheres is listed in Table I. Modified encapsulation protocol was used to prepare microspheres (Silva *et al.*, 2006). In brief, the sodium alginate solution was prepared by dissolving 0.25 g in 30 mL hot water and then TS was dispersed in this solution using a magnetic stirrer for 10 minutes. A suspension of $CaCO_3$ at 5% (w/v) was added to the Alg-TS solution, after homogenization, the mixture was dispersed into paraffin oil (30% internal phase ratio, v/v) containing different concentrations of span 80% as an emulsifying agent and was emulsified by stirring at various speeds. After homogenization for 15 min, 20 mL of paraffin oil containing 0.2 mL glacial acetic acid (acid/Ca molar ratio of 3.5) were added to the w/o emulsion. Mixing was continued for 20 minutes to allow calcium carbonate solubilization and formation of calcium alginate microsphere. After 10 min., a solution of $CaCl_2$ (0.05 M) containing 1% Tween 80 was added to segment and recoup the gelled microspheres. Microspheres were washed with 0.05

M CaCl$_2$ containing 1% Tween 80 to remove residual oil Microspheres were recovered from oily phase by using an acetate buffer at pH 4.5 (USP XXVII) and progressively washed with this buffer until no more oil was detected by optical microscope observation. Chitosan was used as a polymer in the concentration of 0.5, 1 and 1.5%. A chitosan coat was applied by immersing the microspheres into chitosan solution (0.5, 1, 1.5% w: v) containing 0.05 M CaCl$_2$ (pH 5.5) under magnetic stirring. After 30 min., microspheres were washed as described previously.

Production yield determination

The yield of the microspheres was determined in triplicate by dividing the weight of the prepared microspheres by the original amount of the Alg-Ch and TS used and the results were expressed as a percentage according to the equation (Jelvehgari *et al.*, 2010).

%Yield= (Actual weight of microspheres/ Total weight of Alg-Ch and TS) ×100

Particle size determination

The dried microspheres were weighed and sized using USP standard sieve (Rx-86-1, Cole-Palmer Instrument Co., USA). The portion of microspheres remaining on each sieve was gathered and the mean particle size of the microspheres was doled out as the percentage of microspheres retained at each sieve multiplied by the average particle size of the sieve utilized (Sipahigil, Dortunc, 2001). Each investigation was carried out in triplicate.

Determination of drug content

Digestion method was used to determine TS content in the prepared microspheres (Pandey, Khuller, 2004) and the experiments were carried out in triplicate. One hundred micrograms of TS microspheres were crushed carefully in a glass mortar and a definite weight was transferred to a 100 mL volumetric flask using phosphate buffer pH 7.4 and completed to the volume using phosphate buffer pH 7.4. The flask was agitated for 5 min each hour for 5 h. The sample was filtered TS concentration was determined spectrophotometrically at 324 nm (Spectrophotometer UV. 1601, Shimadzu Co., Japan). The plain formula was used as a blank and treated with the same procedures.

Microspheres morphology

The morphology of the microsphere surfaces was investigated using scanning electron microscopy. Microspheres were spread on a carbon double-adhesive layer on a metal holder and gold-coated using an Ion-Sputtering device (Jeol Fine-Coat JFC 1100E, Joel Ltd., Tokyo, Japan). The microspheres were scanned by Scanning Electron Microscope (SEM) (Jeol JSM-5400 LV, Jeol Ltd., Tokyo, Japan).

In-vitro release of TS microspheres

Dissolution testing of the prepared microspheres equivalent to 100 mg of TS was performed with the rotating basket apparatus according to USP 24 apparatus 1 (SR11 6 Flask, Hanson Co., USA). Hard gelatin capsules No. 2 filled with a known quantity of TS microspheres were used for dissolution study using basket speed of 50 rpm and a temperature of 37 °C ± 0.5. Regarding the dissolution medium, fifty mL of phosphate buffer pH 7.4 was used as the release medium for eight hours (Pasparakis, Bouropoulos, 2006). Three milliliters of each sample were removed at specific intervals throughout the whole 8 hrs. The specimens were diluted properly with the dissolution medium and absorbance was measured at the foreordained λ max of each medium against a blank of this medium. The withdrawn samples were supplanted with equal volumes of the dissolution medium. All experiments were completed in triplicate.

Regression analysis

The targeted response parameters were statistically analyzed by applying one-way ANOVA at 0.05 level in Minitab 17 software (Minitab, Inc. USA, Pennsylvania). The dependent variables were evaluated using the F test and quadratic models of the form $Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1 X_2 + \beta_4 X_1^2 + \beta_5 X_2^2$ were created for each response variable using multiple linear regression analysis (MLRA), where Y is the level of the measured response; β_0 is the intercept β_1 to β_5 are the regression coefficients. X_1 and X_2 stand for the main effects; $X_1 X_2$ is the interaction between the main effects; X_1^2 and X_2^2 are the quadratic terms of the independent variables that were used to simulate the curvature of the designed sample space. A backward elimination procedure was adopted to fit the data into different predictor equations.

The quadratic models generated by regression analysis were used to construct the 3-dimensional graphs in which response parameter Y was represented by a curvature surface as a function of X (Dean, Voss, Draguljić, 2017).

Numerical advancement utilizing attractive quality approach was utilized to find the optimal settings of the independent factors to get the coveted response. A streamlined plan was created by setting imperatives on the independent and dependent variables. The optimized formulation was assessed for the responses and the test esteems acquired were contrasted with those anticipated by the numerical models (Dean, Voss, Draguljić, 2017).

Kinetics of the *in-vitro* release of TS capsules

The kinetic parameters for the *in vitro* release of TS were determined and then analyzed in order to find the proper order of the drug release. Zero and first order kinetics, as well as controlled diffusion or Higuchi diffusion model (Higuchi, 1963).

In-vivo studies

The anti-inflammatory effect of TS in the chosen formulae, formulae F12, F6, which had the best rank order among the prepared formulae and the commercial TS tablet was studied on rats using the paw edema method. The paw edema induced by injection of carrageenan in the paw was used as an example for the anti-inflammatory bioassay test of TS capsules.

Rats were divided into four groups, each group consisting of six animals. Each group of animals received the specified drug product by oral administration. The animals previously injected with carrageenan, the percentage of inhibition of edema size was measured after 2, 4, 6, 8, 10 and 12 hours.

RESULTS AND DISCUSSION

Microspheres Morphology

The microspheres prepared with Alg-Ch copolymers were examined using the light microscope, Figure 1. It can be seen that at D:P ratio1:3 the prepared microspheres are spherical in shape with a smoother surface, more intact surface with smaller pores (Figure 1a). Figure 1(a) reveals that at D:P ratio1:1 there is a hollow cavity for the microspheres by internal gelation, Figure 1(d) is the enlarged view of the outer surface, which reveals the

clear loose network structure with the larger pores (dark area). In contrast to these results, SEM micrograph of Nimodipine loaded alginate bead treated with chitosan the beads had a spherical shape and showed surface cracks probably caused by the method of preparation which is external gelation in addition to partial collapsing of the polymer network during drying(Rajendran, Basu, 2009).
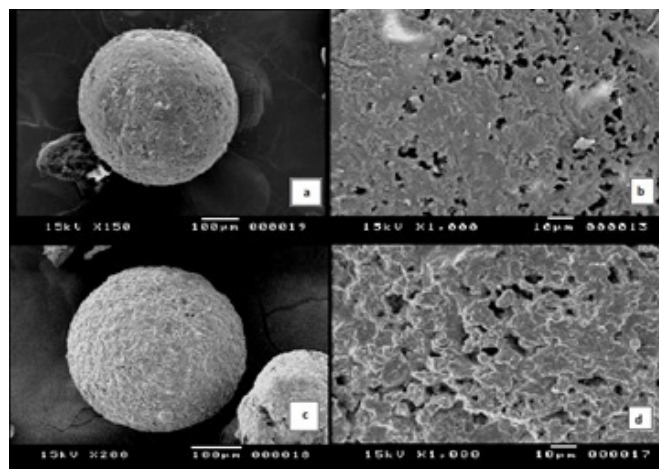
FIGURE 1 - SEM photographs of TS (Ch-Alg) microspheres prepared by internal gelation (a) full view at 1:3 drug to polymer ratio, (b) outer surface at 1:3 drug to polymer ratio, (c) full view at 1:1 drug to polymer ratio, (d) outer surface at 1:1 drug to polymer ratio.

A similar effect on the microspheres shape was stated for salbutamol sulfate microspheres at different ethyl cellulose concentrations (Nath *et al*., 2010). Those particles were spherical in shape and exhibited porous surfaces. The surface of the drug- loaded microspheres manifested the presence of drug particles as compared to blank microspheres. Surface investigation of the prepared TS microspheres after dissolution studies showed bigger pores suggesting that the drug is released through pores and the kinetics of drug release was diffusion controlled mechanism (Motwani *et al*., 2008).

Another study for ibuprofen ethyl cellulose revealed that the microspheres had good spherical geometry as evidenced by the SEM photographs. The surface of the microspheres was quite smooth (van Zwol *et al*., 2008).

Formulae F11, and F15 exhibit the best distribution pattern as the largest weight determined lied between 400 um – 315 um, while The remaining formulae F1, F2, F3, F4, F5, F6, F7, F8, F9, F10, F12, F13, and F14 gave the second group of good distribution as the largest weight calculated lied between 890 um – 630 um . The

particle size analysis of Alg-Ch coated microspheres was done using sieve analysis procedure. The mean particle size values (± SD) for formulations number 1 to 15, were 570.8±(35.1), 549.9±(41.5), 500.2±(39.7), 490.2±(39.8), 509.9±(42.6), 599.8±(32.9), 491.3± (45.2), 466.8± (47.9), 429.9 ± (43.1), 470.1± (39.2), 417.4± (37.9), 578.3± (45.2), 506.8± (47.9), 492.3± (45.2), 402.8± (47.9) µm respectively So, the formulated TS microspheres were arranged according to the mean particle size, in descending order, as following: F6, F12, F1, F2, F5, F13, F3, F14, F7, F4, F10, F8, F9, F11 and F15. High stirring speed (600 rpm) produced microspheres with small particle size while low stirring speed (200 rpm) produced microspheres with large particle size. Similar results were obtained by chitosan coated soya oil containing lipophilic marker as well as plain Ch-Alg microspheres prepared by different crosslinking techniques (Ribeiro et al., 1999). Various manufacturing parameters (apparatus design, type of stirrer, stirring speed, the viscosity of emulsion phase, polymer concentration and emulsifier concentration) affect the particle size of the prepared microspheres (Xu et al., 2007).

D:P ratio markedly affected the size of the microspheres at the three different Alg-Ch concentrations (Zhang et al., 2011). The mean particle size of the prepared microspheres increased with increasing the amount of the copolymers. No clear relationship was found between the particle size of the microspheres and the change in polymer concentrations (Xu et al., 2007). Stirring speed is the most important parameter for controlling the drug/matrix dispersion's droplet size in the continuous phase. It was shown that increasing the stirring speed generally results in decreased microsphere size, as it produces smaller emulsion droplets through stronger shear forces and increased turbulence. The same results were obtained in the microencapsulation of ibuprofen with eudragit RS100 (Perumal, 2001).

Microspheres production yield

The percentage of production yields are listed in Table II. There was no obvious difference between the yield of different formulations on varying both the D:P ratio and the speed.

TABLE II - Production yield, actual drug content and % EE of different TS Alg-Ch microspheres

Formulation number	Yield (%)	Theoretical drug content (%)	Actual drug content (%) Mean ± SD	Loading efficiency (%)
1	94.37±3.11	50.00	37.93±1.51	75.87
2	87.62±4.23	50.00	41.32±2.59	82.64
3	85.14±4.02	50.00	45.15±1.08	90.31
4	88.06±5.08	50.00	49.66±1.99	99.32
5	95.36±4.32	33.33	31.74±1.85	95.25
6	92.80±3.98	33.33	24.25±2.04	72.76
7	83.82±4.51	33.33	26.37±0.94	79.13
8	78.40±4.11	33.33	29.40±1.26	88.23
9	88.33±5.44	33.33	28.32±1.66	84.99

(continuing)

TABLE II - Production yield, actual drug content and % EE of different TS Alg-Ch microspheres

Formulation number	Yield (%)	Theoretical drug content (%)	Actual drug content (%) Mean ± SD	Loading efficiency (%)
10	86.82±4.51	33.33	32.36±2.59	97.11
11	80.55±3.24	33.33	28.96±2.59	86.91
12	89.07±4.43	25.00	19.83±2.59	79.33
13	93.41±3.84	25.00	17.72±2.59	70.89
14	86.29±5.84	25.00	16.74±2.59	66.98
15	90.30±4.44	25.00	23.13±2.59	92.54

Differential scanning calorimetry (DSC)

The drug may have been dispersed in crystalline or amorphous form or dissolved in the polymeric matrix during the formation of microspheres. Any abrupt or drastic change in the thermal behavior of either the drug or polymer may indicate possible drug-polymer interaction. The endothermic peak of the pure drug was observed at about 159.31°C (Figure 2). However, in the thermogram of the microspheres, (containing Alg and Ch) there was an endothermic peak of the drug melting with a lower intensity than the pure drug peak, suggesting the crystalline state of the drug in the microspheres. The DSC showed the stable character of TS in the drug-loaded microspheres and revealed the crystallinity form. Similar results were observed with Nimodipine microspheres prepared by ionic gelation technique (Rajendran, Basu, 2009).
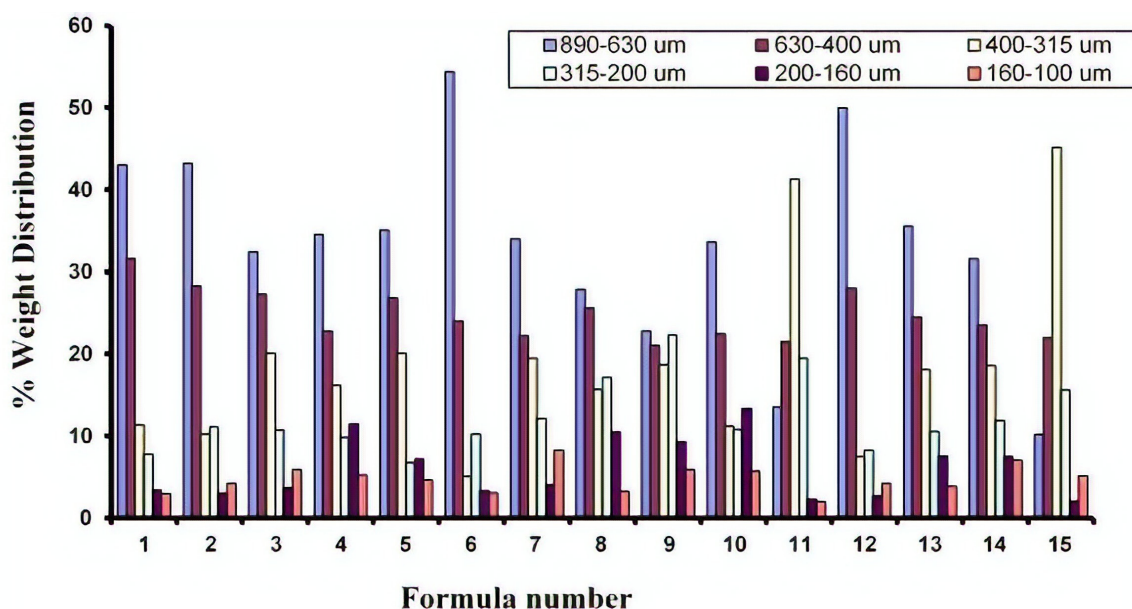
FIGURE 2 - Histogram representing the fraction percent of % weight distribution of different formulae of TS coated Alg-Ch microspheres.

Encapsulation Efficiency Percent (% EE)

The data obtained in the case of TS Ch-Alg microspheres are listed in Table II. According to D:P ratio, the calculated theoretical drug content value for formulations, number 2, 4 and 6 was 33.33%, while for formulations, number 1, 5 and 9 was 25% and for formulations number 3,7 and 8 was 20%. Thus, the loading efficiency values ($\pm$ SD) for the nine formulations were computed as 88.4$\pm$ (4.56), 78.7$\pm$ (5.19), 75.6$\pm$ (4.32), 90.7$\pm$ (3.98), 81.5 $\pm$ (5.57), 79.2$\pm$ (4.09), 78.1$\pm$ (3.79), 92.3$\pm$ (5.04), and 74.8$\pm$(4.99) %. The effect of changing the D:P ratio on the drug loading efficiency was investigated, at each chitosan concentration, using the ANOVA test. TS % EE values depended on the speed of rotation and were found to range from 74.8$\pm$3.67 to 92.3$\pm$1.88. The output shows the results of fitting a multiple linear regression model to describe the relationship between D: P ratio and % EE. The equation of the fitted model is:

$$\% \ EE = 84.12 + 6.58 \ X_1 - 4.80 \ X_2 - 3.62 \ X_3 + 6.48 \ X_1^2 - 3.83 \ X_2^2 - 2.58\% \ X_3^2 - 2.56 \ X_1X_2 + 3.07 \ X_1X_3 - 2.89 \ X_1 \ X_3$$

TABLE III - Factors combinations and response parameters of TS Alg-Ch microspheres prepared with Box-Behnken design

| Formula No.	Variable level in coded form			Cumulative percent release		
	X1	X2	X3	%LE(%w/w)[a]	Rel$_2$[a]	Rel$_8$[a]
F1	-1	-1	0	75.87	16.46	76.56
F2	0	-1	-1	82.64	23.43	97.54
F3	0	-1	+1	90.31	20.34	94.70
F4	+1	-1	0	99.32	22.56	90.65
F5	-1	0	-1	95.25	18.77	70.65
F6	-1	0	+1	72.76	16.40	62.76
F7	0	0	0	79.13	22.99	81.76
F8	0	0	0	88.23	19.50	68.41
F9	0	0	0	84.99	20.22	78.21
F10	+1	0	-1	97.11	23.87	82.76
F11	+1	0	+1	86.91	22.88	85.34
F12	-1	+1	0	79.33	14.54	62.76
F13	0	+1	-1	70.89	21.57	73.54
F14	0	+1	+1	66.98	23.32	48.26
F15	+1	+1	0	92.54	20.76	66.34

The parentheses in the data represent the decoded factor levels. X_1 represents drug to polymer ratio; X_2 represents speed, X_3 represents a span 80%. % LE = Percentage loading efficiency; Rel$_2$ = Release at the end of 2 h; Rel$_8$ = Release at the end of 8 h.
[a]The values represent the average of three determinations (n = 3)

Since the P-value in the ANOVA table is greater or equal to 0.10, there is not a statistically significant relationship between the variables at the 90% or higher confidence level. The R-Squared statistic indicates that the model as fitted explains 0.188324% of the variability in D:P ratio. On the other hand, the output shows the results of fitting a multiple linear regression model to describe the relationship between speed and % EE. Since the P-value in the ANOVA table is less than 0.01, there is a statistically significant relationship between the variables at the 99% confidence level. The R-Squared statistic indicates that the model as fitted explains 87.0527% of the variability in speed. The model indicated that the speed emerged as the lone factor, which exerted a significant influence on % EE.

The 3-D plot, Figure (3) showed that %EE increased from 75.87±1.12 to 99.32±0.62 and from 79.33±1.61 to 92.54±1.15 at lower and higher levels of speed with a constant D:P ratio. The improvement in %EE with increased speed levels was due to the fact that more drug gets dissolved and incorporated in the polymer matrix with a high speed of rotation. A linear relationship between the two variables investigated on the percent drug incorporation was clearly seen from the corresponding contour plots which suggested that %EE can be enhanced using a high speed of rotation (Figure 4). Microencapsulation by the emulsification/ internal gelation method involves two major steps, the formation of stable droplets of the polymer solution with drug incorporated in as an emulsified system and the subsequent solidification of the droplets. These two steps have a significant effect on the size and encapsulation efficiency of microspheres. Similar results were obtained in the preparation of biodegradable paclitaxel loaded alginate microspheres for pulmonary delivery (Alipour, Montaseri, Tafaghodi, 2010).
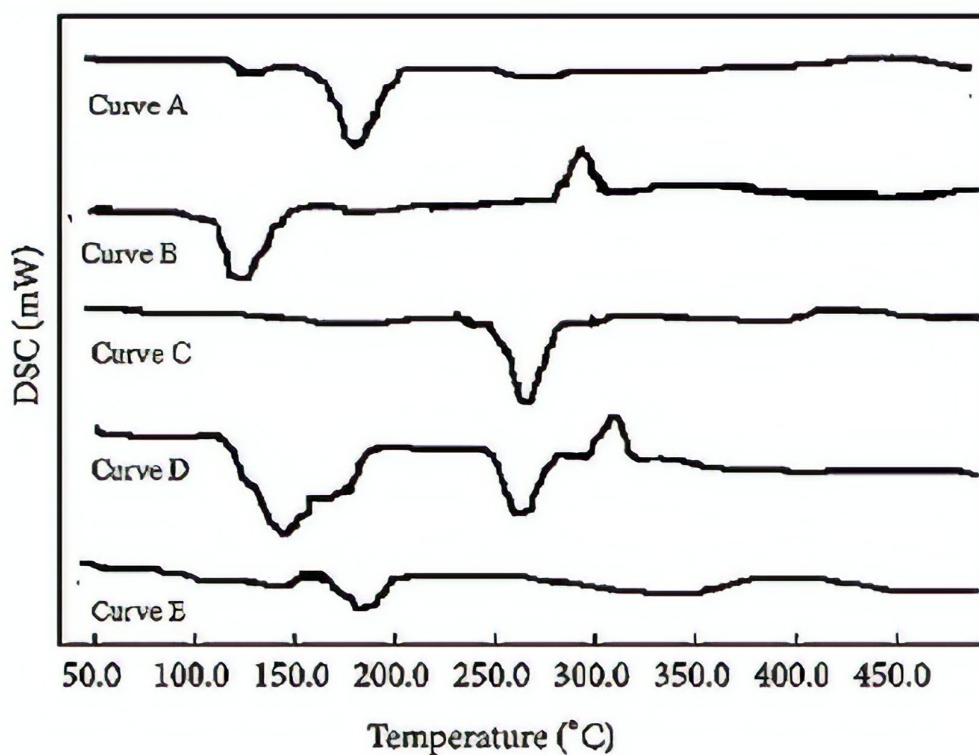
FIGURE 3 - Differential Scanning Calorimetry of TS (curve A), chitosn (curve B), sodium alginate (curve C), physical mixture (Curve D) and the prepared microspheres (curve E).
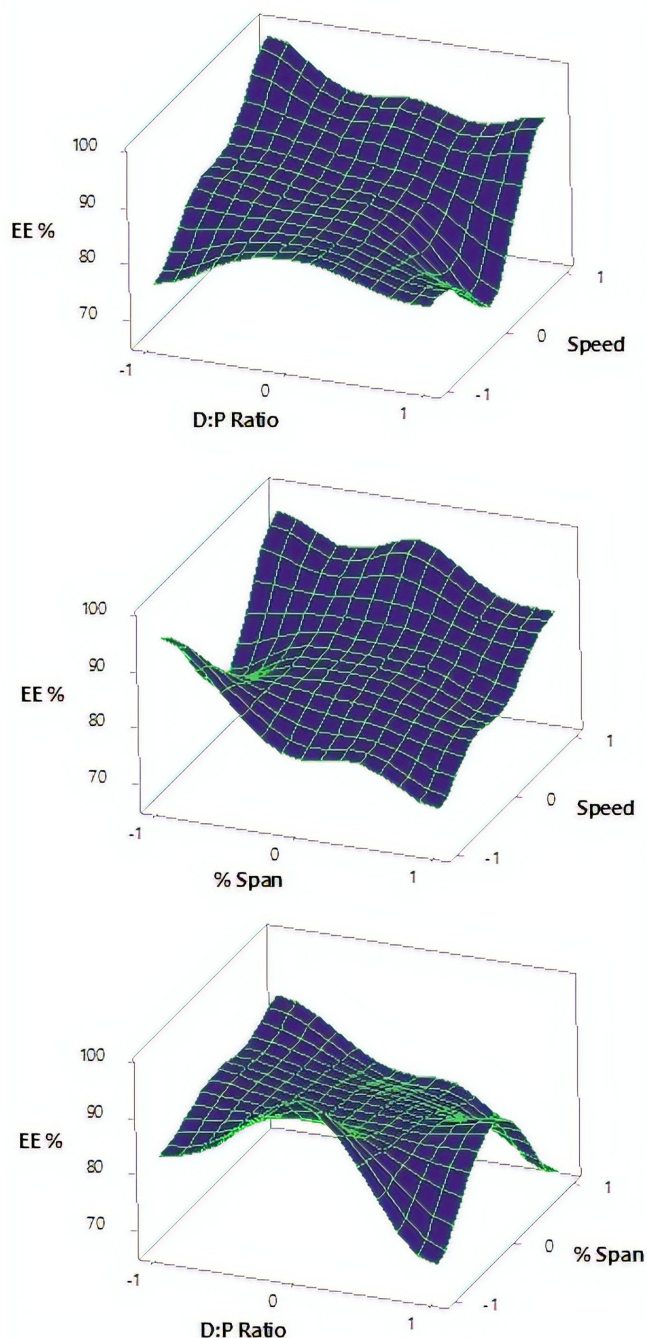
FIGURE 4 - Three-dimensional surface response plot for the effect of speed (X_1), D: P ratio (X_2) and span 80% (X_3) on the drug loading efficiency (Y_1).

In-vitro release of TS microspheres

Figure 5 shows the *in-vitro* release of TS from its capsules containing formulae (F1, F2, F3, and F4) using constant drug: polymer ratio 1:1 (X_1) with variable speed of rotations, 200 rpm for F1; 400 for F2,3 and 600 rpm for F4; (X_2). The maximum and minimum percent released was

observed to be 23.43% and 16.46% at the end of two hours (Y_2). The maximum and the minimum in-vitro release after eight hours (Y_3) of dissolution were found to be equal to 97.54±3.32% and 76.56±1.76%, respectively. These investigated formulae can be arranged, in descending order, concerning the *in- vitro* release within eight hours as follows: F2 > F3 > F4 > F1 respectively.
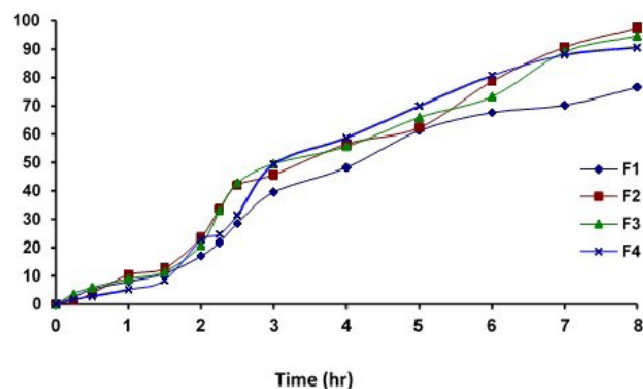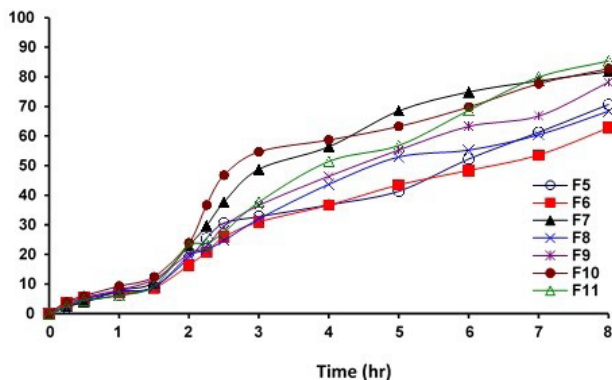

FIGURE 5 - *In-vitro* release of chitosan-coated TS Alg-Ch capsules containing drug: polymer ratio 1:1.

Figure 6 show the *in-vitro* release of TS from its capsules containing formulae F5, F6 F7, F8 F9, F10 and F11 using D:P ratio1:2 (X_1) with variable speeds, 200 rpm for F5, F6; 400 rpm for F7, F8, F9 and 600 rpm for F10, F11 (X_2). The maximum and minimum percent released was observed to be 23.87±1.09% and 16.40±0.98% at the end of two hours (Y_2). The maximum and minimum *in-vitro* release after eight hours of dissolution (Y_3) was found to be 85.34±2.21% and 62.76±2.02%. According to these results, these formulae can be arranged, in descending order, concerning the *in-vitro* release within 8 hours dissolution as follows: F11 > F10> F7> F9> F5 > F8> F6 respectively.

The *in-vitro* release of TS from its capsules containing formulae F12, F13, F14, and F15 using constant drug-polymer ratio 1:4 (X_1) with variable speeds, 200 rpm for F12; 400 rpm for F13, F14 and 600 rpm for F15 (X_1) Figure (7). The maximum and minimum percent released was observed to be 23.32±1.12 and 14.54±1.14 after the end of two hours (Y_2). The maximum and minimum *in-vitro* releases after eight hours (Y_3) of dissolution were found to be 73.54±2.67 and 48.26±2.03%. According to these results, these formulae can be arranged, in descending order, concerning the *in-vitro* release within 8

hours dissolution as follows: F13 > F15> F12> F14 respectively.

FIGURE 6 - *In-vitro* release of chitosan-coated TS Alg-Ch capsules containing drug: polymer ratio 1:2.
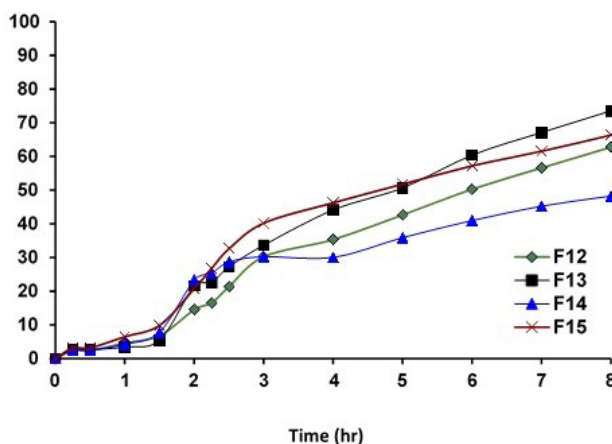
FIGURE 7 - *In-vitro* release of chitosan-coated TS Alg-Ch capsules containing drug: polymer ratio 1:3.

The best formulae for the *in-vitro* release after the whole dissolution period (8 hours) were observed to be F14, F12, and F6. The equation of the fitted model which study the effect of D:P Ratio and speed on drug release after 2 hours is

$$Y(2hrs)=20.903+2.988X_1-0.325X_2-0.588X_3-2.004X_1^2-0.319\ X_2^2+1.581\ X_3^2+0.030\ X_1\ X_2+0.345\ X_1\ X_3+.210\ X_2X_3$$

Since the P-value in the ANOVA table is less than 0.01, there is a statistically significant relationship between the variables at the 99% confidence level. The R-Squared statistic indicates that the model as fitted

explains 98.5588% of the variability in release 2 hours. The drug-polymer ratios were found to have a negative influence on the drug release whereas the speed had a positive influence on the drug release.

The response surface plots (Figure 8) illustrate that the drug release at the end of two hours decreased from 16.46±1.54% to 14.54±1.23% and from 22.56±1.89 to 20.76±1.42% at low and high levels of the drug to polymer ratio, respectively as the polymer content increased. This can be due to better incorporation of the drug in the polymeric matrices, which left behind fewer drug crystals on the surface with the increase in the polymer content.

The levels of speed were found to have a positive influence on the drug release after two hours. It was evident from the 3-D plots (Figure 7) that the drug release at the end of two hours increased from 16.46±1.21% to 22.56±1.32% and from 14.54±1.14% to 20.76±1.12% at low and high speed levels respectively. This increase in drug release was due to the decrease in particle size of prepared microspheres which increased the surface area available for drug diffusion and consequently increased the permeability of Alg-Ch copolymers. These results indicated that drug release could be minimized using low levels of speed at high polymer levels.

The equation of the fitted model which study the effect of drug-polymer ratio and speed on drug release after 8 hours is:

$$Y\ 8hrs = 76.13 + 6.55\ X_1 - 13.57\ X_2 - 4.18\ X_3 - 2.59\ X_3^2 + 0.54\ X_2^2 + 1.84\ X_3^2 - 2.63\ X_3X_2 + 2.62\ X_3X_1 - 5.61\ X_2\ X_3$$

Drug-polymer ratio and speed had a significant effect on drug release at the end of 8 hours with an F value of 165.35 ($p < 0.0001$). The R-Squared statistic indicates that the model as fitted explains 98.218% of the variability in 8 hours release. The drug-polymer ratios were found to have a negative influence on the drug release whereas the speed had a positive influence on the drug release.

The response surface plots (Figure 9) illustrate that the drug release at the end of eight hours decreased from 76.56±1.76% to 62.76±2.03% and from 90.65±3.32 to 66.34±2.67% at low and high levels of D:P ratio, respectively as the polymer content increased. This can be due to better incorporation of the drug in the polymeric matrices, which left behind fewer drug crystals on the surface with an increase in the polymer content.
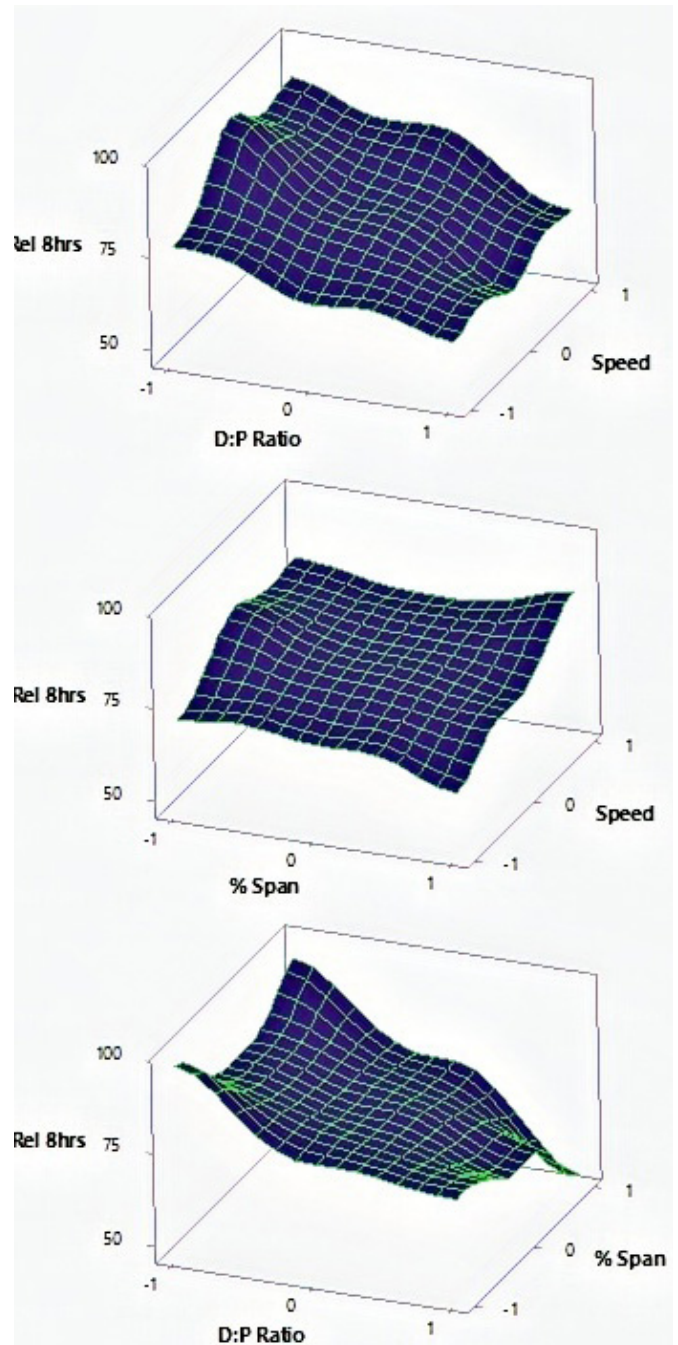
gradient established between the interior of the microspheres and the release medium.
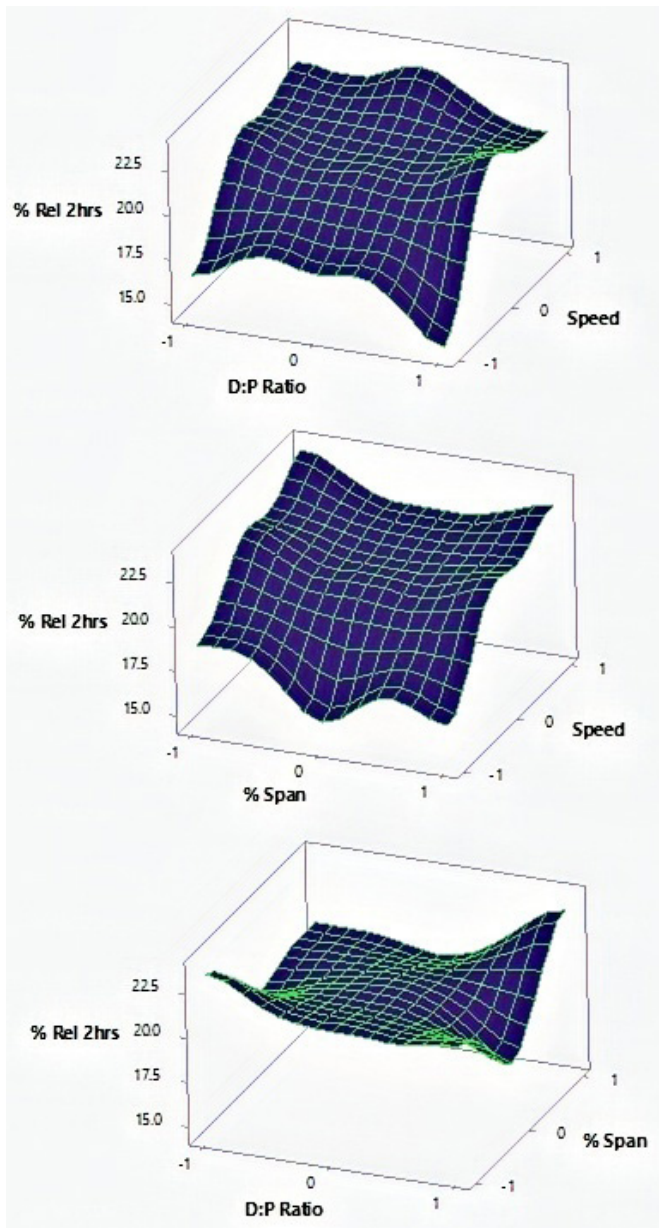
FIGURE 8 - Three-dimensional surface response plot for the effect of D: P ratio (X_1) and speed (X_2) on drug release after two hours (Y_2).

The release data of TS from Alg-Ch microspheres prepared with different process variables were analyzed according to zero and first-order kinetics as well as, Higushi diffusion-controlled mechanism Table 4. The release order of the drug was determined. It was found that the drug was released from the microspheres according to the zero-order pattern, at different drugs to polymer ratios. The release medium dissolves the drug after penetrating the microspheres wall by allowing the dissolved drug to diffuse out due to concentration

FIGURE 9 - Three-dimensional surface response plot for the effect of D: P ratio (X_1) and speed (X_2) on drug release after eight hours (Y_3).

TABLE IV - Analysis of the release data for TS microspheres prepared at a different drug: polymer ratios of sodium Alg-Ch mixture

| Formula number		F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	F15
Zero-order	K_z	8.148	10.50	7.108	8.837	8.781	9.482	6.44	9.323	9.323	8.735	6.44	10.50	9.323	9.323	9.323
	R_z	0.984	0.991	0.968	0.981	0.984	0.989	0.961	0.986	0.986	0.982	0.961	0.991	0.986	0.986	0.986
First-order	K_f	0.139	0.291	0.127	0.171	0.167	0.217	0.104	0.203	0.203	0.271	0.104	0.291	0.203	0.203	0.203
	R_f	0.962	0.942	0.952	0.960	0.958	0.966	0.948	0.963	0.963	0.930	0.948	0.942	0.963	0.963	0.963
Higuchi equation	K_h	21.26	18.70	33.09	29.24	19.98	23.75	22.16	31.69	31.69	28.24	22.16	18.70	31.69	31.69	31.69
	R_h	0.924	0.990	0.967	0.976	0.971	0.985	0.960	0.976	0.976	0.936	0.960	0.990	0.976	0.976	0.976

K_z (mg.hr^{-1}), K_f (hr^{-1}) and K_h (mg/cm^2. hr$^{1/2}$) are the release rate constants of zero–order, first order and, Higuchi model kinetics, respectively, as well as R_z, R_f and R_h are their corresponding correlation coefficients.

In-vivo studies

The anti-inflammatory effect of TS microspheres and the commercial tablet on the size of edema induced in the right hind paw of rats induced by carrageenan (Onishi *et al.*, 2010). Treatment of the rats with TS microspheres and tablet significantly inhibit the edema size induced by carrageenan injection in the intra-plantar area of the right hind paw at the therapeutic dose level for the rats 2 mg/kg) (Solanki *et al.*, 2015). It was observed that group two (treated with commercial tablet) produced the maximum percent edema inhibition at 8 hours and the effect decreased gradually with time up to 10 hours. Regarding the anti-inflammatory activity of TS microspheres, F12 and F6 of TS-Alg-Ch showed the highest inhibitory effect at 8 hours after the injection of carrageenan (percentage of edema inhibition was 62.71 and 58.76%, respectively).

These results are illustrated in Figure 10. The maximum anti-inflammatory activity of the selected formulation higher than that of TS commercial tablet. After ten hours both groups still showing the anti-inflammatory activity. The ANOVA of the results of the anti-inflammatory activity of TS formulation on edema size. From this table, we noted that as a whole set of experiment, all the investigated formulae were

significantly inhibiting edema size and the p-value less than 0.05 were considered as significant.
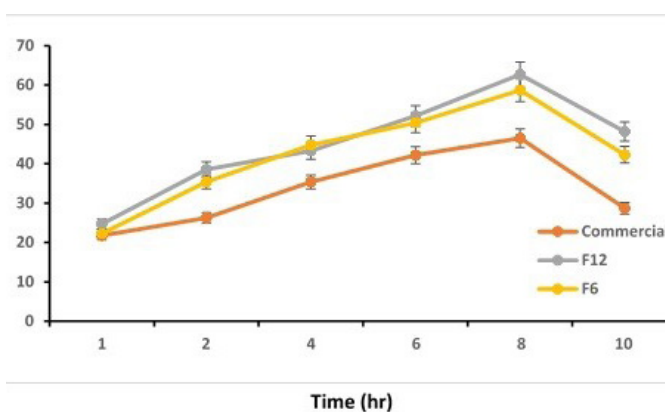
FIGURE 10 - Effect of TS formulations on the inhibition of rat hind paw edema induced by carrageenan.

CONCLUSION

Coating the alginate microspheres with chitosan delayed the drug release by forming Alg-Ch complex. The microspheres were spherical in shape with large pores at D: P ratio1:1 while at 1:3 D:P ratio formed microspheres

having a smooth surface and small pores. The change in chitosan concentration had a non-significant effect on drug EE %. Increasing Ch concentration decreased the drug release significantly. The increase in the speed of rotation had significantly decreased drug loading efficiency, while it is significantly increased the drug release. TS Alg-Ch microspheres followed zero-order release kinetics. The selected formulae TS microspheres give a significant inhibition of rat paw edema. The behavior of the TS Alg-Ch microspheres is of great interest for the delivery of the non-steroidal anti-inflammatory drug into the small intestine.

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