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Formulation and Evaluation of Mucoadhesive Microspheres for Nasal Drug Delivery

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ABSTRACT

Terbutaline sulphate loaded microspheres were prepared for Nasal administration with the aim of avoiding first pass effect. The microspheres were prepared by a water-in-oil (w/o) emulsification-cross-linking technique by using chitosan as a mucoadhesive polymer, liquid paraffin (heavy and light, ratio 1:1) as a external phase, dioctyl sodium sulfosuccinate (0.2% w/v) as a stabilizer, volume of cross-linking agent (Glutaraldehyde, 25% solution, 1 mL) and time of cross linking 2 hrs. A 2^3 full factorial design was constructed to study the effect of three independent variables i.e. drug: polymer ratio (X_1), volume of cross linking agent (ml) (X_2), cross linking time (Hrs)(X_3) while Particle size of the microspheres (Y_1) and In vitro mucoadhesion (Y_2) were taken as response parameters as the dependent variables. Particle size was found to be 26.11 ± 1.98 mm, which is favorable for intranasal absorption. The shape and surface characteristics were determined by scanning electron microscopy (SEM) which depicted the spherical nature and nearly smooth surfaces of the microspheres. The percentage encapsulation efficiency was found to be 74.4 ± 0.604%. In vitro mucoadhesion was performed using sheep nasal mucosa and was observed 72.32 ± 0.25%. FTIR Spectroscopy indicates characteristic peaks of the functional groups present in the drug, Differential scanning calorimetry and X-ray diffraction results indicated a molecular level dispersion of Terbutaline sulphate in the microspheres. *In vitro* release studies in pH 6.2 phosphate buffer indicated the mechanism of drug release was of zero order.

Keywords: Terbutaline, sulphate, mucoadhesive microspheres, emulsification-cross-linking technique, Chitosan, factorial design.

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INTRODUCTION

Nasal drug delivery is a useful delivery method for drugs that are active in low doses and show no or minimal oral bioavailability.[1]Bioadhesive application of liquids, semisolids or solids formulations to the nose has been explored with respect to deposition and retention. In addition, it was reported that combinations of bioadhesive polymers with permeation enhancers would further improve nasal bioavailability[2]. Nasal drug absorption is affected by molecular weight, size, formulation, pH, pKa of the molecule, and delivery volume. Among other formulation characteristics, molecular weight still presents the best correlation to absorption[3, 4]. The apparent cut-off point for molecular weight is 1, 000 with molecules less than 1, 000 having better absorption. Shape is also important. Linear molecules have lower absorption than cyclic shaped molecules[5]. Additionally, particles should be larger than um, otherwise the drug may be deposited in the lungs. Hydrophillicty has also been found to decrease drug bioavailability[6].

Mucoadhesive Drug Delivery System

Mucoadhesive are synthetic or natural polymers, which interact with the mucus layer covering the mucosal epithelial surface and mucin molecules constituting a major part of mucus. The concept of mucoadhesive has alerted many investigators to be possibility that these polymers can be used to overcome physiological barriers in long-term drug delivery. They render the treatment more effective and safe, not only for topical disorders but also for systemic problems[7]. Mucoadhesive controlled release devices can improve the effectiveness of a drug by maintaining the drug concentration between effective and toxic levels, inhibiting the dilution of a drug at a specific site. Mucoadhesion also increases the intimacy and duration of contact between a drug containing polymer and a mucous surface. The combined effects of the direct drug absorption and decrease in excretion rate (due to prolong residence time) allow for an increased bioavailability of the drug with a smaller dosage and less frequent administration. Bioadhesive system can prevent the first pass metabolism of certain protein drugs by liver through the introduction of the drug via route bypassing the digestive tract. Drugs that are absorbed through the mucosal lining of tissues can enter directly into the blood stream and prevented from enzymatic degradation in the GIT[8].

Nasal Mucoadhesive Drug Delivery System

Nasal therapy known as “Nasaya Karma” has been recognized in Ayurvedic medicine since ages. However, the potential of nasal drug delivery was recognized in year 1992. Conventionally the nasal route has been used for the delivery of drugs in the treatment of local diseases; however the last decade has recognized the importance of the nasal cavity as potential route for drug delivery.

There are an increasing number of research and review articles addressing topics on nasal drug delivery. This interest arises from the different possible advantages presented by the nasal cavity. 13-22

Mucoadhesive Microspheres

Mucoadhesive microspheres include micro particles and microcapsules (having a core of the drug) of 1-1000µm in diameter and consisting either entirely of a bioadhesive polymer or having an outer coating of it, respectively. Microspheres in general are investigated for targeted and controlled release drug delivery. A polymeric device allows for slow, controlled, and predictable drug release over a period of time and hence reduces the overall amount of drug needed. In nasal drug delivery, coupling of bioadhesive properties to microspheres is of great importance because of additional advantages: efficient absorption and enhanced bioavailability of the drug, a much more intimate contact with mucus layer and reduction in frequency of drug administration due to the reduction in mucociliary clearance of drug delivery system adhering to nasal mucosa[26].

Application of mucoadhesive microspheres

Mucoadhesive microspheres have been extensively studied for a number of applications (Figure 8). Majority of these can be understood by classifying these applications on the basis of routes of administration[27].

Advantages of Nasal Drug Delivery

1) Rapid drug absorption and quick onset of action
2) Avoidance of first pass metabolism
3) Avoidance of irritation of gastrointestinal membrane
4) Avoidance of degradation of drugs in the gastrointestinal tract, resulting from acidic or enzymatic degradation[21,29]

MATERIALS AND METHOD

Pure drug Terbutaline sulphate obtain from Blue Cross Ltd. Nashik as a gift sample, Chitosan were purchased from Central Institute of Fisheries Technology, Cochin / Mumbai, Glutaraldehyde solution 25%, Dioctyl sodium sulfo succinate , Acetic Acid, Liquid paraffin (Heavy & Light), Sodium chloride, Sodium hydroxide, Potassium dihydrogen phosphate and Hydrochloric acid were purchase from SD Fine Chemie Pvt. Ltd. Mumbai. Other essential machineries, sophisticated instruments, and experimental setup were provided by Vidyabharti College of pharmacy, Amravati.

Method of Formulation

Chitosan microspheres were prepared by simple w/o emulsification-cross-linking process describe by Thanoo et al., 1992 using liquid paraffin (heavy and light, 1:1) as external phase. The hardened microspheres were separated by Remi centrifuge and washed several times with hexane to remove oil. Finally, microspheres were washed with distilled water to remove unreacted GA. The microspheres were air dried for 8 hrs and then stored in vacuum desiccator until further use.

Table 1: Formulation Table

Formulation code	Drug: polymer ratio	Volume of GA	Cross linking time (Hrs.)	Particle size (µm)	Product yield (%)	DEE (%)*	DL (%)*	(%) In vitro mucoadhesion
CM1	1:1	1	2	21.75 ± 0.774	80.35%	62.4 ±0.230	31.2± 0.075	63.25 ± 1.04
CM2	1:2	1	2	26.80 ± 1.447	86.4%	74.4 ±0.604	24.8±0.226	71.92 ± 0.61
CM3	1:3	1	2	31.13 ± 0.603	79.2%	61.86 ±0.262	15.46±0.00	73.89 ± 0.54
CM4	1:4	1	2	38.55 ± 2.12	74.32%	56.01 ±0.628	11.2±0.075	75.12 ± 1.27
CM5	1:5	1	2	48.468 ± 0.612	72.1%	55.2 ±0.239	9.2±0.226	76.30 ± 0.66

Evaluation of Chitosan Microsphere

Particle size

Particle size diameter is one of the important parameter for evaluating the particle size of microspheres. Particle size was determined according to standard procedure.[31] using Optical microscope.

Photomicrographs

Photomicrographs of drug loaded chitosan microspheres were taken by using Optical microscope at 10X magnification.

Scanning Electron Microscopy (SEM)

Shape and surface characteristics of optimized formulation F2 were studied by scanning electron microscopy (JSM 5610 LV, Jeol Datum Ltd., Japan) with 10 kv accelerating voltage. The sample were mounted directly onto the SEM sample holder using double- sided sticking tape and images were recorded at the required magnification at the acceleration voltage of 5kV.

Product yield

The yields of production of microspheres of various batches were calculated using the weight of final product after drying with respect to the initial total weight of the drug and polymer used for

preparation of microspheres and percent production yields were calculated as per the formula mentioned below[32]

Product yield = Practical mass (microspheres) /Theoretical mass (polymer + drug) X 100 (1)

Drug entrapment efficiency and Drug loading

The weighed amount of microspheres were dissolved in distilled water and kept overnight. The drug content was measured spectrophotometrically at 276 nm for TB. The drug loading and incorporation efficiency (%) were calculated by using "equations (2)-(3)" respectively.

Drug loading (%) $= M_{\text{actual}}$ /Weighed quantity of powder of microspheres× 100…….... (2)

Entrapment efficiency (%) = $M_{\text{actual}} / M_{\text{theoretical}} \times 100$

Where M_{actual} is the actual Terbutaline sulphate content in weighed quantity of powder of microspheres and $M_{\text{theoretical}}$ is the theoretical amount of Terbutaline sulphate in microspheres calculated from the quantity added in the preparation process.[33]

Swelling property

Microspheres were suspended in 10 ml of phosphate buffer saline pH 6.2. the particle size of the microspheres was monitored in each medium every one hours by optical microscope and swelling index of microspheres was calculated. The swelling index was calculated from the following expression

Swelling index =Final particle size – Initial particle size/ Initial particle size (4)

***In vitro* mucoadhesion study**

The mucoadhesive property was determined by falling liquid film technique. A freshly cut piece, 5 cm long of sheep nasal mucosa obtained from a local abattoir within 2 hrs of killing the animal, then the mucosa was cleaned by washing with isotonic saline solution. Accurately weighed quantity of microspheres was attached over a polyethylene plate. This plate was kept in desiccator to maintain at > 75 % relative humidity and room temperature for 30 minutes to allow the polymer to interact with the membrane and also to prevent drying of the mucus. Then this plate was attached to the outer assembly at an angle of 45º relative to the horizontal plane, and pH 6.2 phosphate buffer saline warmed at 37^0C was peristaltic ally pumped at a rate of 5 ml/min over the tissue. After 1 hour of administration, the concentration of the drug in the collected perfusate was spectrophotometrically determined at 276nm. The adhered microspheres amount was estimated from the difference between the applied microspheres and the flowed microspheres amount. The ratio of the adhered microspheres was computed as percent mucoadhesion[34].

***In vitro* drug release studies**

The drug release profile of different formulations of Terbutaline sulphate loaded chitosan microspheres were evaluated using Franz diffusion cell apparatus which consisted of donor and receptor compartment. The treated cellophane membrane was fixed between the donor and receptor compartment of the diffusion cell to keep the sprinkled microspheres on the donor side which allow free diffusion of Terbutaline sulphate to the receptor compartment containing 20 ml phosphate buffer saline solution pH 6.2(within the pH range in nasal cavity) and maintained at 37 ±1°. The receptor compartment was stirred with magnetic stirring bar. The samples were withdrawn periodically for 8 h and replaced with same volume of fresh phosphate buffer saline solution (pH 6.2). The samples were analyzed spectrophotometrically for drug content at 276 nm[35].

Optimization of microspheres formulation utilizing 2^3 factorial design

A 2^3 full factorial design was constructed to study the effect of three independent variables i.e. drug: polymer ratio (X_1), volume of cross linking agent (ml) concentration (X_2), cross linking time (Hrs)(X_3) and two level. Particle size of the microspheres (Y_1) at *In vitro* mucoadhesion (Y_2) were taken as response parameters as the dependent variables.

Drug permeation through mucosal membrane

Drug permeation through mucosal membrane was assessed using sheep nasal mucosa for ensuring *in vivo* drug absorption. The mucosal membrane was placed between the donor and receptor compartment of the Franz diffusion cell. Weighed quantity of microspheres were sprinkled on the donor side which allow free diffusion of Terbutaline sulphate to the receptor compartment containing 20 ml phosphate buffer saline solution pH 6.2(within the pH range in nasal cavity) and maintained at 37±1°. The receptor compartment was stirred with magnetic stirring bar. The samples were withdrawn periodically for 8 h and replaced with same volume of fresh phosphate buffer saline solution (pH 6.2). The samples were analyzed spectrophotometrically for drug content at 276 nm[36].

X-ray diffraction study

X-ray diffractogram pattern of the plane Terbutaline sulphate, placebo chitosan microsphere and drug loaded microspheres were recorded using X-ray diffractometer (Bruker AXS D8 Advance, with X-ray source of Cu, Wavelength 1.5406 A^0 and Si (Li) PSD detector). Which was operated at the voltage 40 kV and the current 35 mA. These studies are useful to investigate crystallinity of drug in the microspheres. The samples were mounted on a sample holder and XRD patterns were recorded in the 2θ angle range between 3^0 and 89^0 and 0.020 step size at the speed of 5°/min.

Stability study

Stability study of any pharmaceutical formulation is important to study the degradation of drug in formulation. Microspheres improve the stability of the many drugs, as the drug is coated with the polymer. The stability of chitosan microspheres was carried at40 ± 2^{o} C and 75 % ± 5% Relative humidity for period of 1 month. The microsphere batches of optimized formulation were kept in vial, tightly closed. At the interval of 1 week, sample was withdrawn from vial for one month and samples were analyzed for the drug content &Percentage Drug release at each time. [35, 37]

RESULTS AND DISCUSSION

Preformulation Study

A. Description

It is white or almost white crystalline powder in appearance. It has some odour or almost odorless.

B. Melting point

The melting point of the pure drug was found within the reported limits between 270^{0}C - 272^{0}C.

C. Spectroscopic study

1. UV spectrophotometric study

Experimental conditions

Wavelength (nm): 276.0 nm

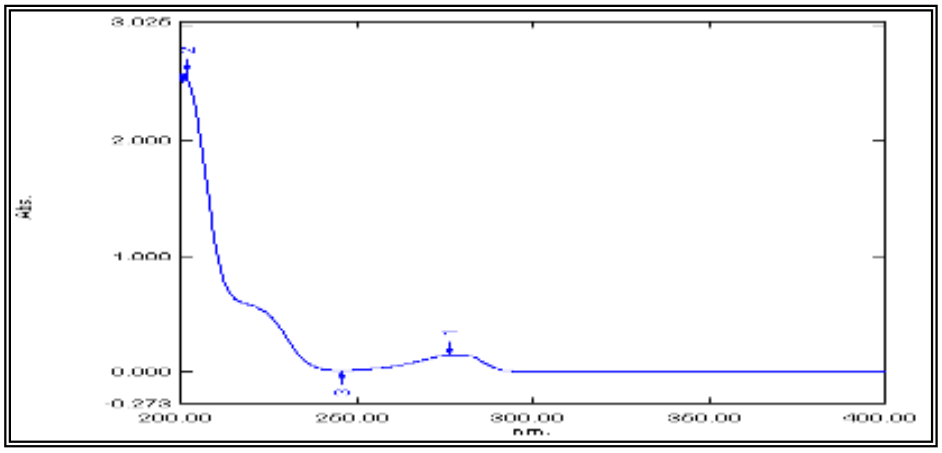


Figure 1: UV Spectrum of Terbutaline sulphate in phosphate buffer (pH 6.2)

Drug and polymer compatibility studies

A. FT-IR Spectroscopy

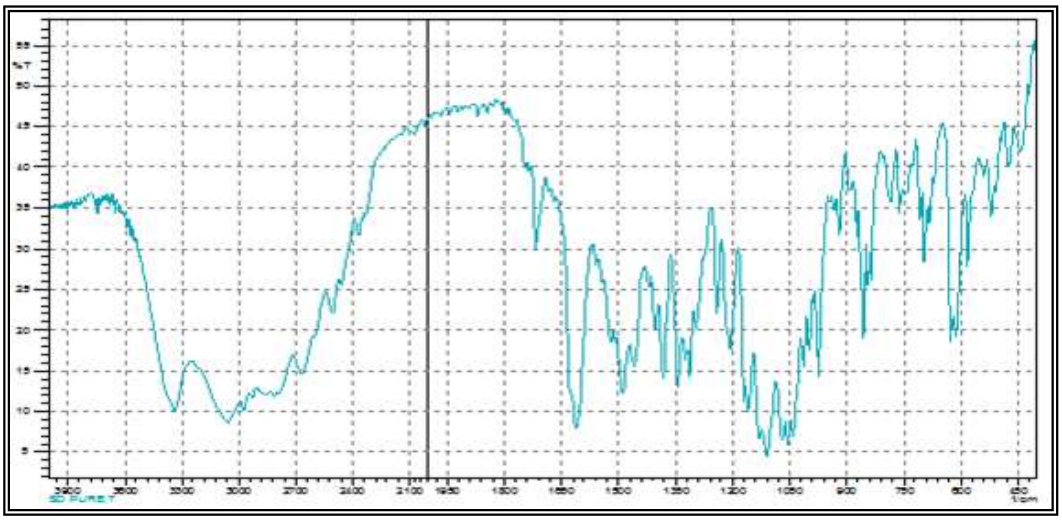


Figure 2: IR spectra of pure Terbutaline sulphate

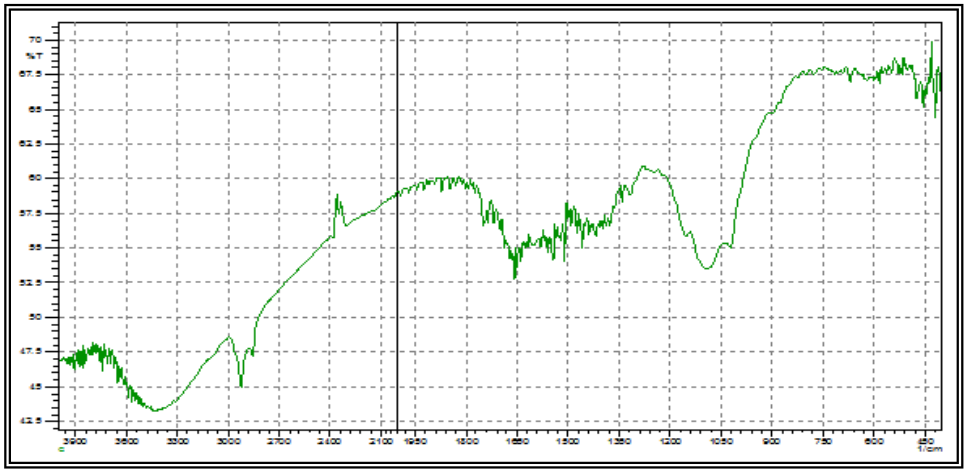


Figure 3: IR spectra of chitosan

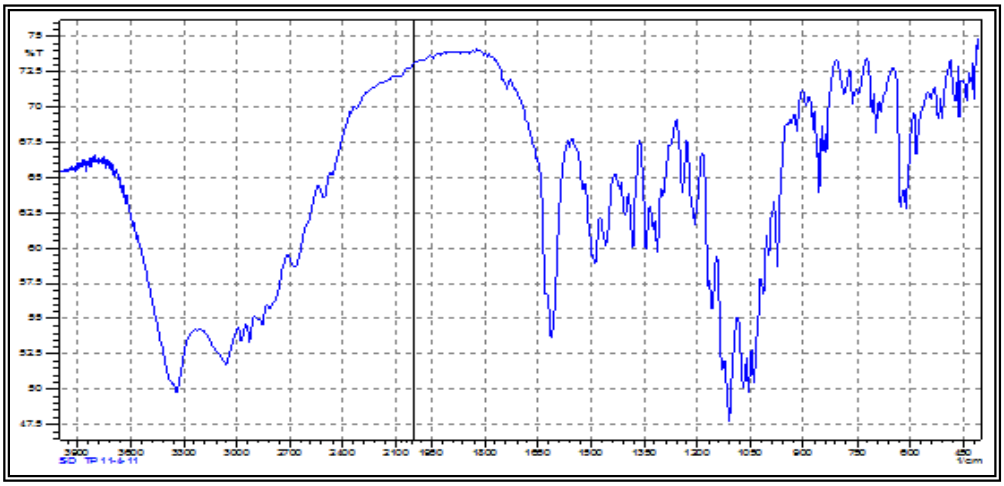


Figure 4: IR spectra of physical mixture of Terbutaline sulphate with chitosan

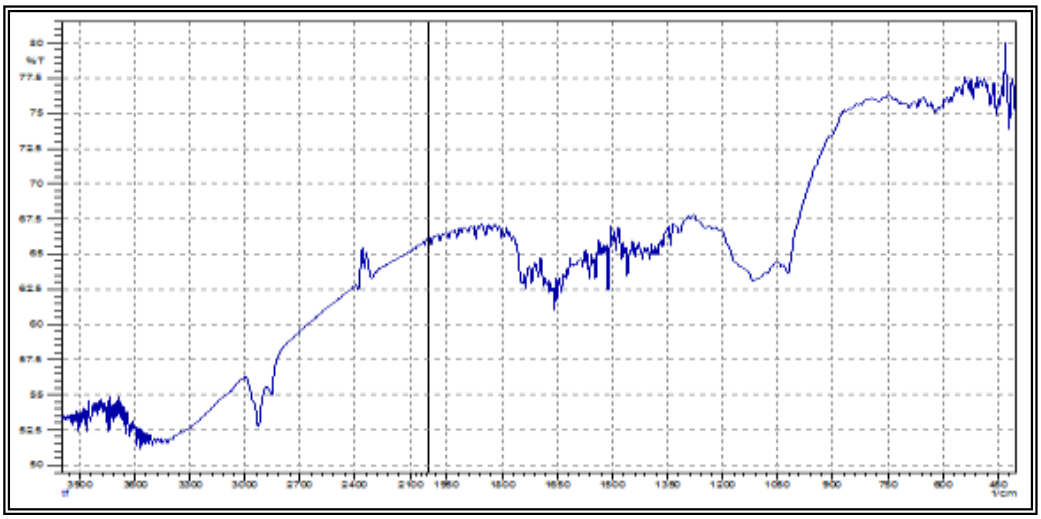


Figure 5: IR spectra of Terbutaline sulphate loaded microspheres

B. Differential scanning calorimetry

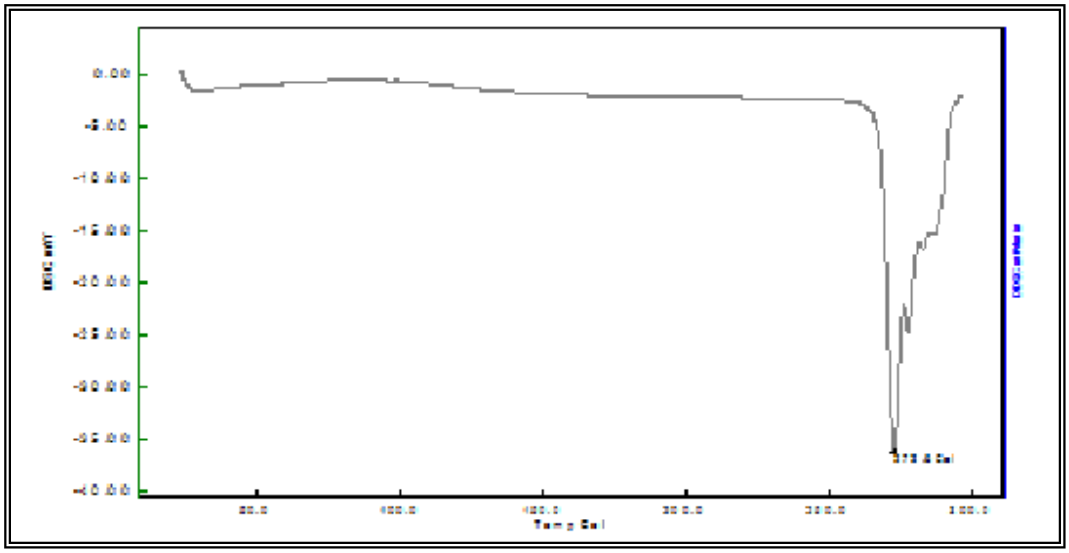


Figure 6: DSC thermogram of pure Terbutaline sulphate

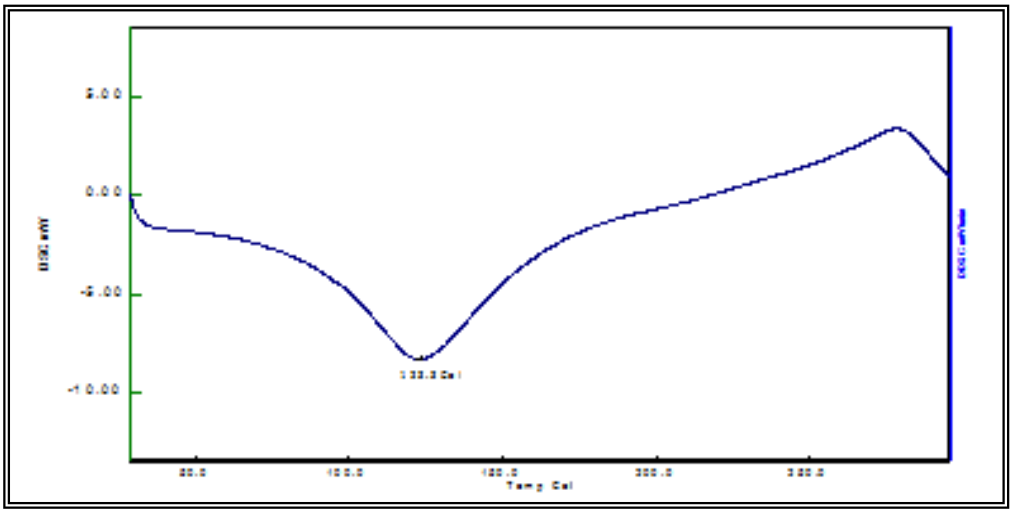


Figure 7: DSC thermogram of blank microspheres

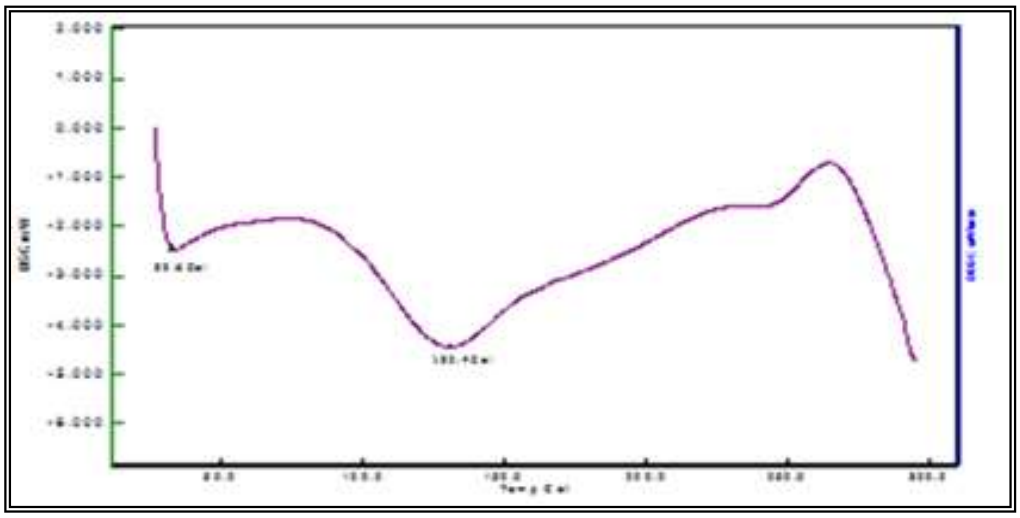


Figure 8: DSC thermogram of drug loaded microspheres

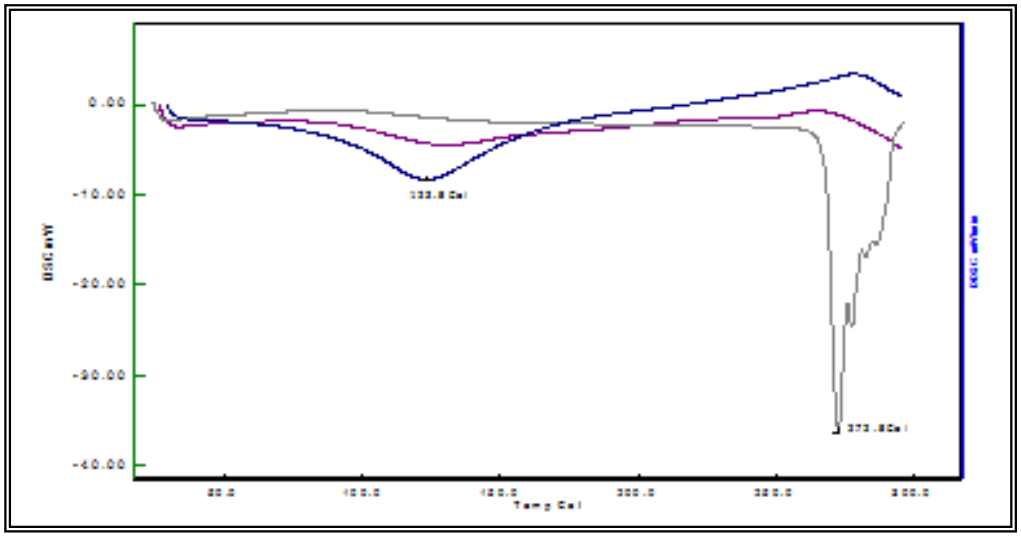
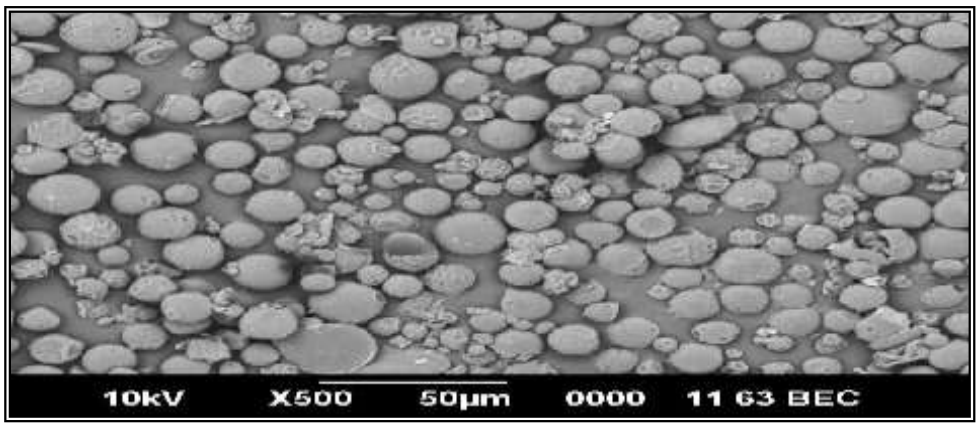
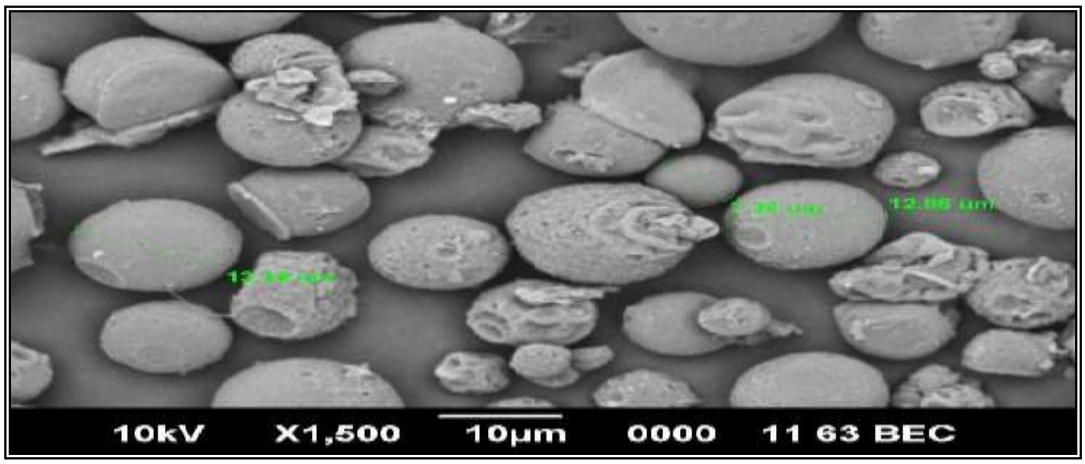


Figure 9: DSC thermogram of pure Terbutaline sulphate, blank microspheres, drug loaded microspheres (overlay graph)

Scanning Electron Microscopy (SEM)



(A)



(B)

Figure 10: SEM Photographs of drug loaded microspheres

Table 2: Characterization of Metformin HCl microspheres

Particle size, product yield, drug encapsulation efficiency, drug loading

Formulation code	Particle size (µm)	Product yield (%)	DEE (%)*	DL (%)*
CM1	21.75 ± 0.774	80.35%	62.4 ± 0.230	31.2± 0.075
CM2	26.80 ± 1.447	86.4%	74.4 ± 0.604	24.8± 0.226
CM3	31.13 ± 0.603	79.2%	61.86 ± 0.262	15.46± 0.00
CM4	38.55 ± 2.12	74.32%	56.01 ± 0.628	11.2± 0.075
CM5	48.468 ± 0.612	72.1%	55.2 ± 0.239	9.2± 0.226

*Values expressed as Mean± SD; n=3.

Swelling property

Swelling is one of the most important characteristic of mucoadhesive microspheres. It mainly affects the mucoadhesion and also affect release rate from the polymeric system. Form Table 19. The swelling index of microspheres increased as polymer concentration was increased. For batches CM1 to CM5, the degree of swelling was observed from 0.91% to 1.072%.

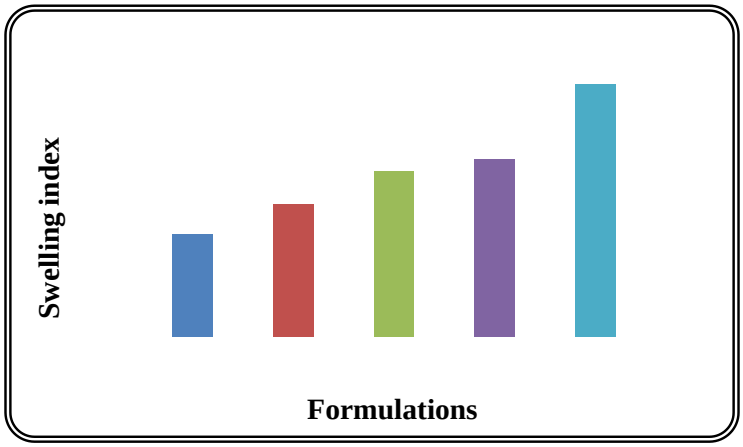


Figure 11: Graph of swelling index of drug loaded chitosan microspheres

***In vitro* mucoadhesion study**

Table 3: Percent mucoadhesion of drug loaded chitosan microspheres

Formulation code	(%) *In vitro* mucoadhesion
CM1	63.25 ± 1.04
CM2	71.92 ± 0.61
CM3	73.89 ± 0.54
CM4	75.12 ± 1.27
CM5	76.30 ± 0.66

* Values expressed as Mean ± SD, n=3

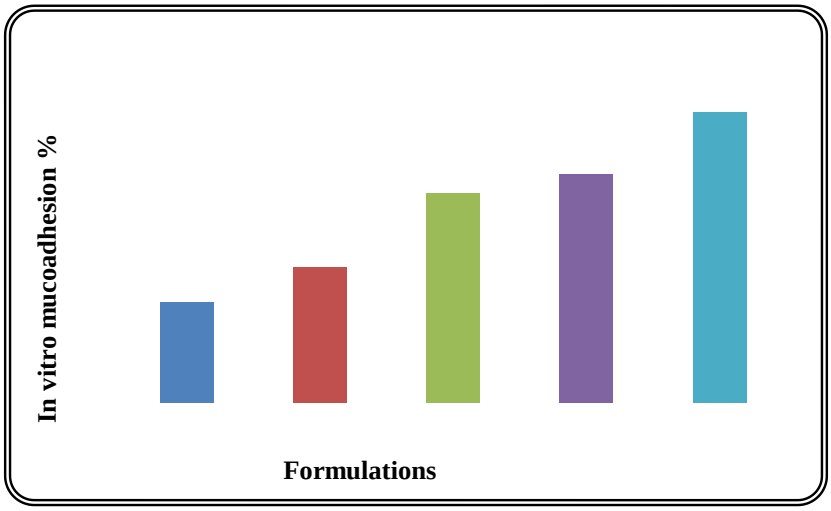


Figure 12: *In vitro* mucoadhesion for batches CM1- CM5

***In vitro* drug release profile**

Table 4: *In vitro* drug release profile of Terbutaline sulphate microspheres Formulations

Time (Hrs)	% Cumulative Drug release				
	CM1	CM2	CM3	CM4	CM5
0	0	0	0	0	0
1	14.4 ± 0.28	15.6 ± 0.38	15.3 ± 0.65	12.9 ± 0.51	12.01 ± 0.17
2	18.49 ±0.57	22.3 ±0.24	19.75 ±0.50	17.19 ±0.31	16.81 ± 0.69
3	25.77 ±0.74	31.01 ±0.28	25.6 ± 1.02	23.11 ±0.74	22.9 ± 0.17
4	33.71 ±1.02	40.55 ±0.14	33.81 ±0.78	31.21 ±1.32	29.57 ± 0.58
5	41.11 ±0.34	50.19 ±0.75	40.61 ±0.65	38.2 ± 0.51	37.91 ± 0.11
6	51.63 ±0.37	59.75 ±0.17	48.29 ±0.24	44.97 ±0.63	43.95 ± 1.00
7	61.16 ±0.88	67.01 ±0.57	57.39 ±0.78	54.78 ±0.24	55.29 ± 1.24
8	71.29 ±1.23	73.94 ±0.49	67.29 ±0.19	65.23 ±0.69	64.19 ± 0.38

* Values expressed as Mean ± SD, n=3

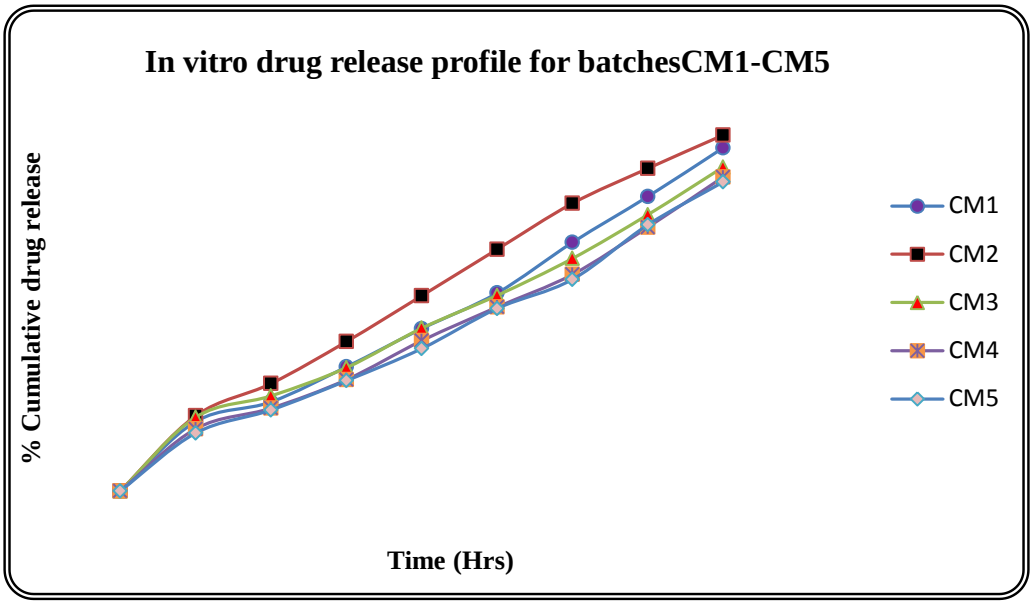


Figure 13: *In vitro* drug release profile for formulations CM1- CM5

Optimization of microspheres formulation utilizing 2^3 factorial design

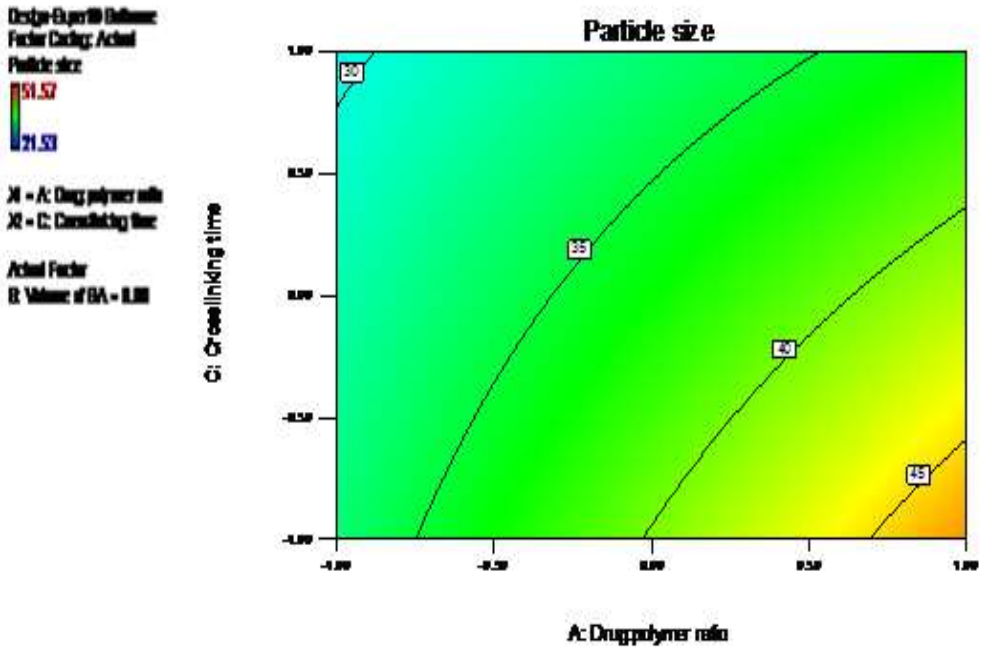
Factorial design parameter and experimental condition

Table 5: Optimization of microspheres formulation utilizing 2^3 factorial design

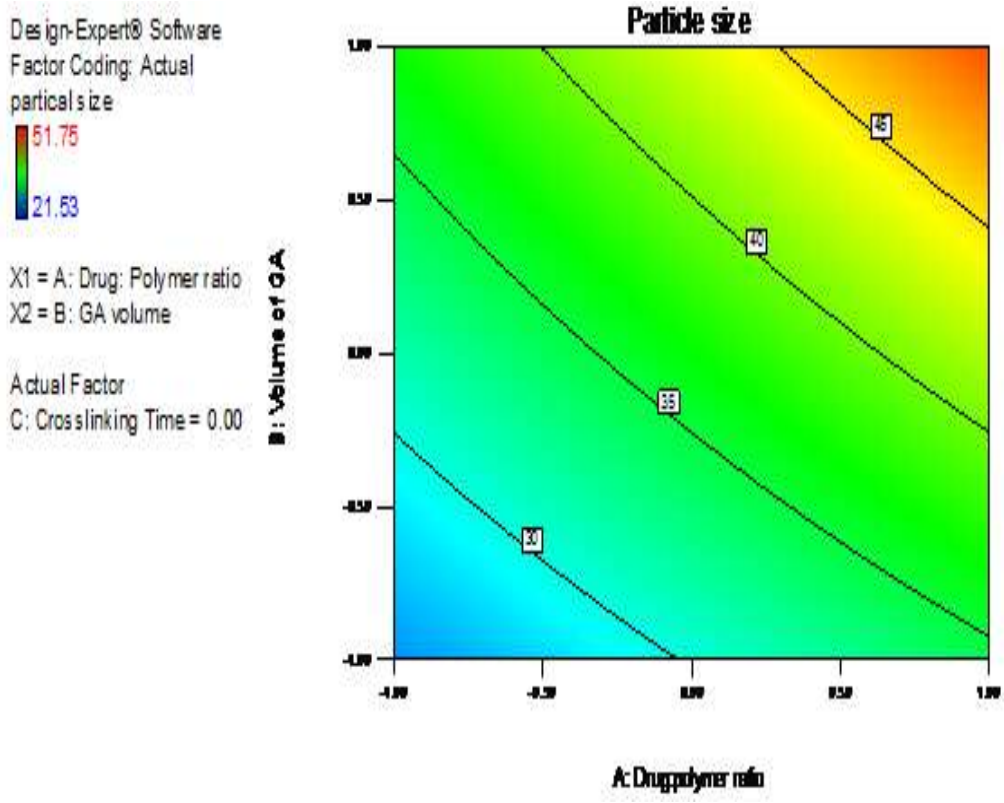
Formulation code	Drug: polymer ratio (X1)	Volume of cross linking agent (ml) (X2)	Cross linking time (Hrs) (X3)	Particle size (µm) (Y1)	In vitro mucoadhesion (%) (Y2)
F1	1:1	2	2	37.64± 2.47	60.68 ± 0.31
F2	1:2	1	2	26.11± 1.98	72.32 ± 0.25
F3	1:2	1	1	42.75± 1.15	65.71 ± 0.21
F4	1:2	2	1	51.57± 0.68	68.92 ± 0.17
F5	1:1	1	2	21.53± 0.72	63.67 ± 0.22
F6	1:2	2	2	47.22± 2.27	69.91 ± 0.14
F7	1:1	1	1	30.36± 0.76	55.39 ± 0.58
F8	1:1	2	1	36.14± 4.24	59.04 ± 0.16

*Values expressed as Mean± SD; n=3

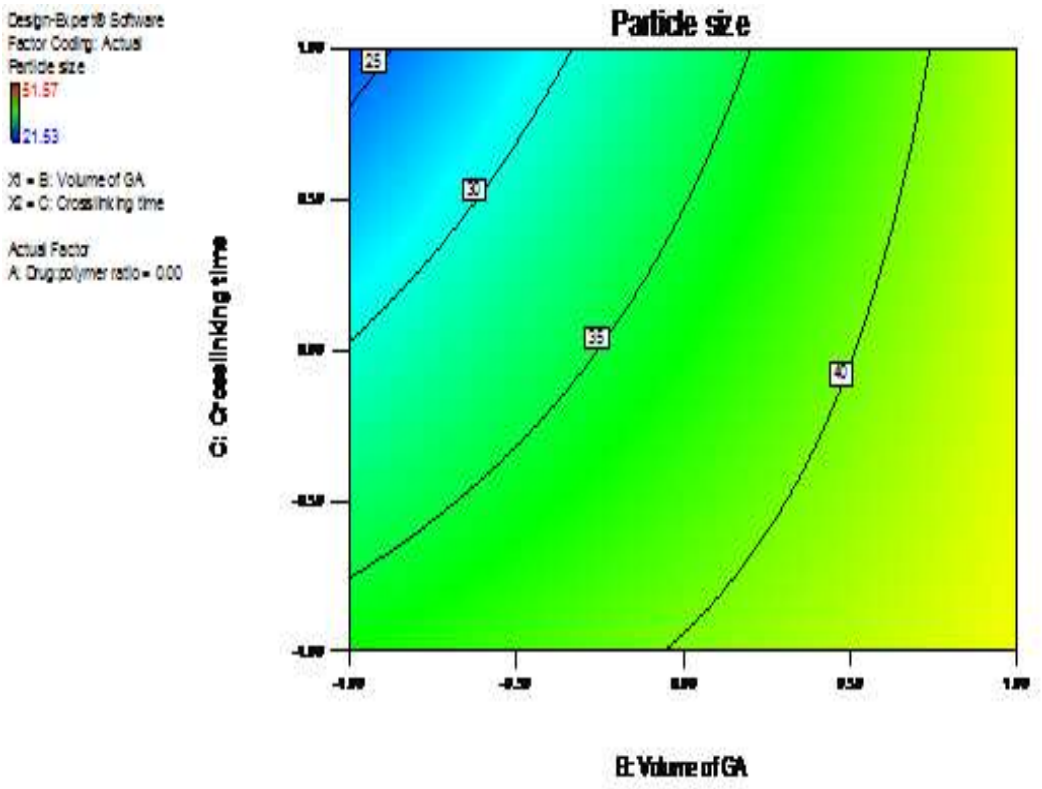
3-D Response curve for Particle size (Y1)


Particle size
51.57
21.53
C: Crosslinking time
A: Drug:polymer ratio
30
35
40
45

(a)

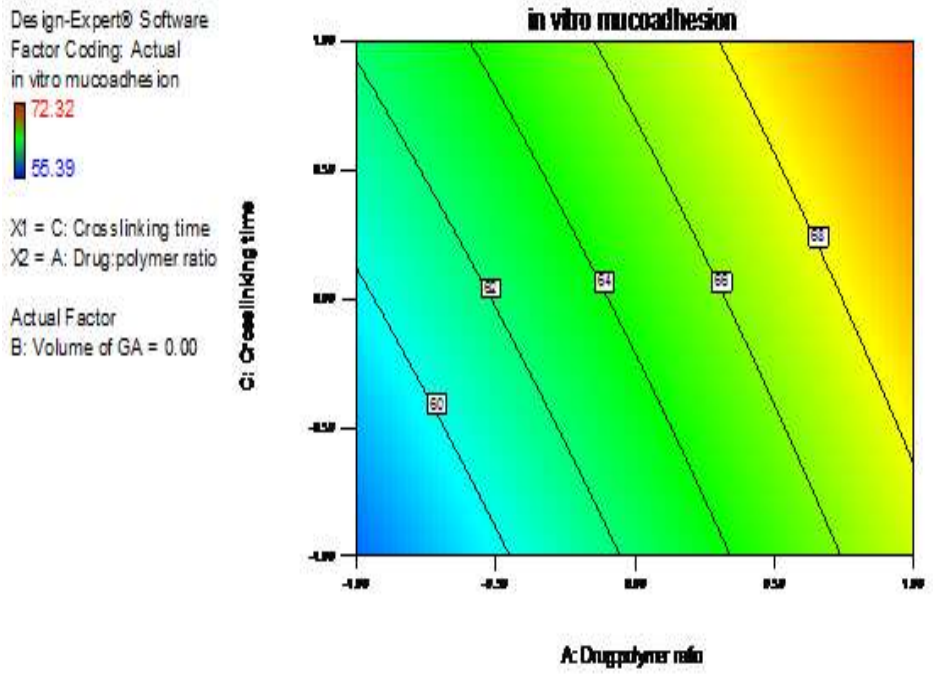

Design-Expert® Software
Factor Coding: Actual
partical size
51.75
21.53
X1 = A: Drug: Polymer ratio
X2 = B: GA volume
Actual Factor
C: Crosslinking Time = 0.00
Particle size
B: Volume of GA
A: Drug:polymer ratio
30
35
40
45

(b)

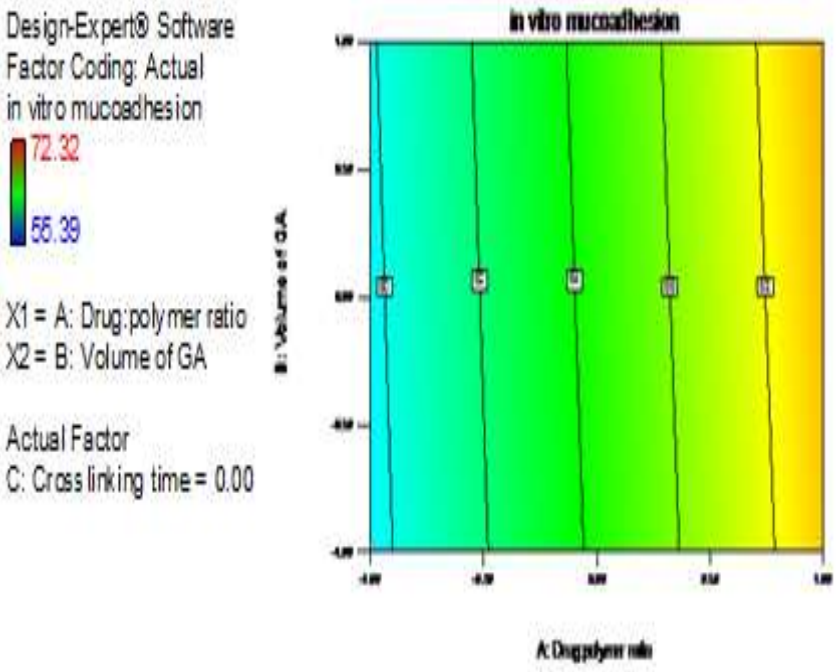


(C)

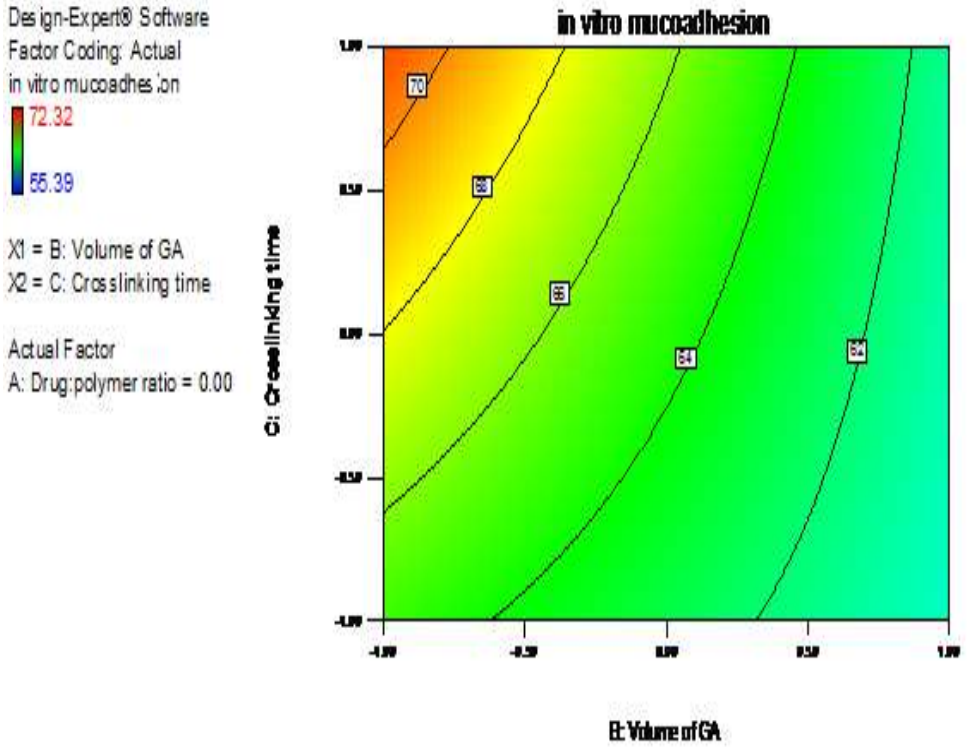
Figure 14: Counter plot for the (a) Effect of drug: polymer ratio (X_1) and crosslinking time (X_3), (b) Effect of drug: polymer ratio (X_1) and volume of crosslinking agent (X_2), (c) Effect of volume of crosslinking agent (X_2) and crosslinking time (X_3) on particle size (Y_1)



(a)



(b)



(c)

Figure 15: Counter plot for the (a) Effect of drug: polymer ratio (X_1) and crosslinking time (X_3), (b) Effect of drug: polymer ratio (X_1) and volume of crosslinking agent (X_2), (C) Effect of volume of crosslinking agent (X_2) and crosslinking time (X_3) on *in vitro* mucoadhesion (Y_2)

From the results of 2^3 factorial design, it was found that as concentration of polymer increased, the *in vitro* mucoadhesion was also increased and the volume of crosslinking agent increases, the mucoadhesive strength of microspheres was decreased. This may be due to that, increase in crosslinking of free-amino groups of chitosan results in decrease in the degree of freedom after some extent and hence reduction in the degree of entanglement.

Flow properties of microspheres

The batches develop as per 2^3 factorial design were evaluated for bulk density, Tapped Density, Compressibility index (%), Angle of repose. according to procedure.

Table 6: Characteristics of prepared Terbutaline sulphate microspheres as per 2^3 factorial design

Formulation code	Bulk density (g/ml)	Tapped Density (g/ml)	Compressibility index (%)	Angle of repose (Degree)
F1	0.33	0.275	16.7 ± 0.866	31.06± 1.12
F2	0.33	0.28	15.3 ± 0.866	29.81± 0.82
F3	0.33	0.275	16.7 ± 0.00	32.12± 1.38
F4	0.34	0.28	17.7 ± 0.577	31.79± 1.29
F5	0.34	0.28	17.7 ± 0.577	29.88± 0.59
F6	0.33	0.28	15.2 ± 0.866	31.92± 1.57
F7	0.33	0.27	18.2 ± 0.288	32.12± 1.03
F8	0.33	0.27	18.2 ± 0.00	32.29± 1.27

The results of flow properties measurements are shown in Table 25. The value of angle of repose were in the range of 29.81± 0.82 - 32.29± 1.27 which is within the normal acceptable range of 20^0-40^0. The microspheres thus shows reasonably good flow potential the value of compressibility index which were in the range 15.2 ± 0.866 -18.2 ± 0.288, also indicating good flow characteristics of microspheres. For formulation F2 angle of repose &compressibility index was 29.81± 0.82 & 15.3 ± 0.866 thus showing good flow property.

***In vitro* drug release study**

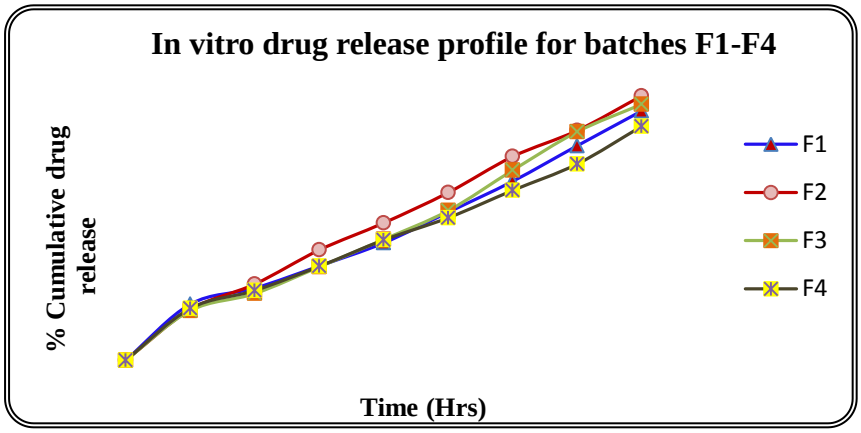


Figure 16: *In vitro* drug release profile for batches F1-F4

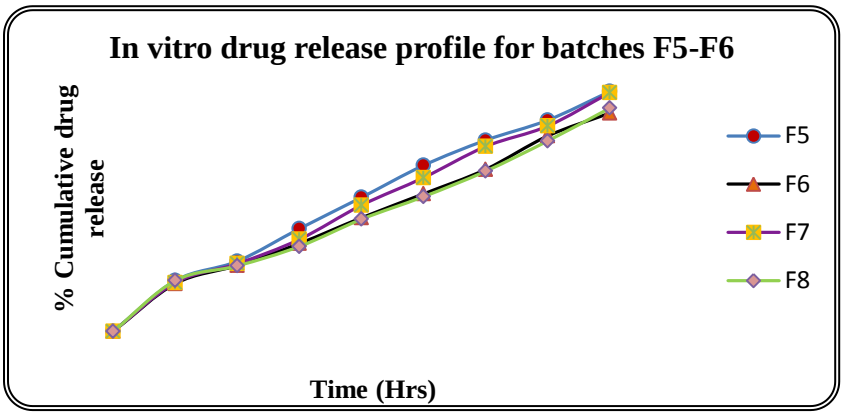


Figure 17: *In vitro* drug release profile for batches F5-F8

Kinetic Modeling of Drug Release

Kinetic models (Zero order, First order, Hixon Crowell, Korsmeyer peppas, Higuchi plot) applied to all developed batches as per 2^3 factorial design.

Table 7: Various parameters of the model equations on the *in vitro* release kinetics

Formulation	Coefficient of regression (R2)				
	Zero order	First order	Hixon Crowell	Korsmeyer peppas	Higuchi plot
F1	0.9923	0.8236	0.9213	0.889	0.9639
F2	0.9973	0.8345	0.9309	0.845	0.9708
F3	0.9931	0.8216	0.9179	0.949	0.9700
F4	0.9939	0.8218	0.9157	0.946	0.9762
F5	0.9953	0.8283	0.9259	0.897	0.9738
F6	0.9949	0.8282	0.9236	0.8720	0.9694
F7	0.9963	0.8339	0.9302	0.8311	0.9685
F8	0.9945	0.8471	0.9407	0.8322	0.9517

The release kinetics of the matrices is shown in table. The best fit model representing the mechanism of drug release was of zero order.

X-ray diffraction study

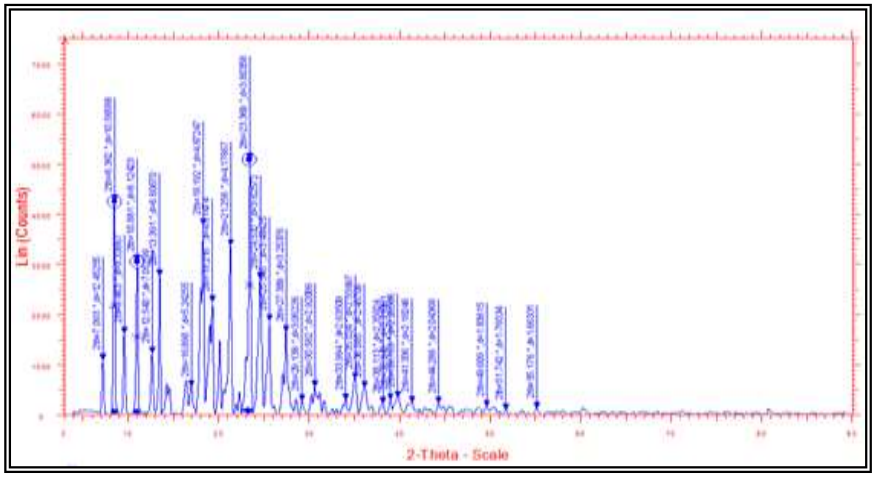


Figure 18: X-ray diffractogram of pure drug Terbutaline sulphate

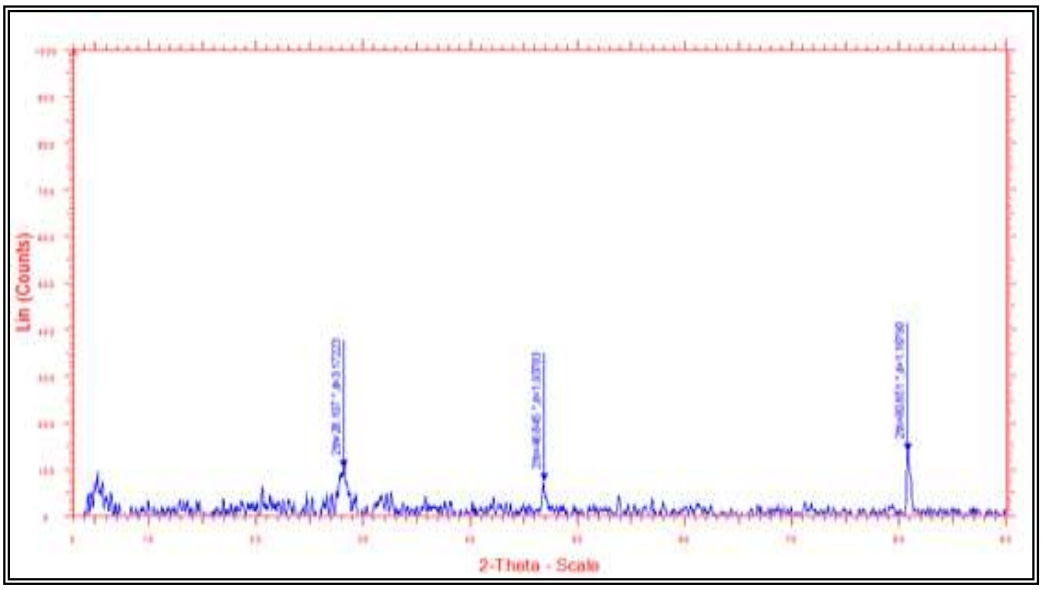


Figure 19: X-ray diffractogram of placebo microsphere

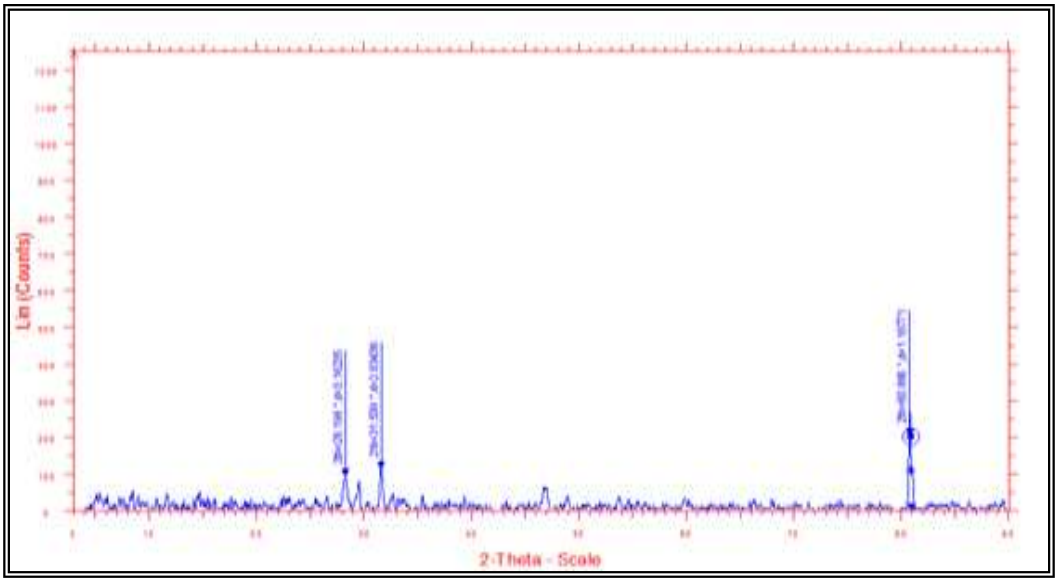


Figure 20: X-ray diffractogram of drug loaded microspheres

CONCLUSION

The chitosan microspheres of Terbutaline sulphate were formulated which aimed at avoidance of first pass metabolism of Terbutaline sulphate, long-acting β2 agonists, used in the treatment of asthma in patients also requiring anti-inflammatory therapy. Mucoadhesive microspheres are attractive concept, in that the drug can be entrapped inside particles to be released at the mucosal surface, where the particles are sticking and the particles can be administered into the nasal cavity. SEM photographs showed that microspheres were almost spherical in shape with slight smooth surfaces. *In vitro* mucoadhesive studies revealed that concentration of polymer in the microspheres, amount of cross linking agent and time of crosslinking affect the *in vitro*

mucoadhesion. It was concluded that as concentration of polymer increased, the in vitro mucoadhesion was also increased. It was concluded that as the time of cross linking and the volume of cross linking agent increases, the mucoadhesive strength of microspheres was decreased. This may be due to that, increase in cross linking of free – amino groups of chitosan results in decrease in the degree of freedom after some extent and hence reduction in the degree of entanglement.

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