Research Article

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Preparation and in-vitro evaluation of mucoadhesive microbeads containing Timolol Maleate using mucoadhesive substances of *Dillenia indica L.*

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Microbeads of Timolol Maleate containing NMS were formulated with the aim of developing new formulation with decreased dose size and therefore redused side effects, control rate and extent of drug release on target site, higher chemical stability, increased residence time due to mucoadhesive property and localization of drug to the specific sites. . The purpose of this work was to prepare controlled release microbeads of Timolol maleate with desired release profile. To achieve that particulate sodium alginate and NMS were used to prepare sustained release mucoadhesive microbeads of Timolol maleate. To design the microbeads by inotropic gelation method and investigate the shape and size of the various beads by microscopic studies. To study the effect of drug-mucoahesive polymer ratio, type and concentration of crosslinking agent ($CaCl_2$, $BaCl_2$, $Al_2(SO_4)_3$, stirring speed and curing time on drug entrapment, swelling index, mucoadhesiveness and *in vitro* release profile in phosphate buffer p^H 6.8 for "pre- gasitric absorption". To investigate the effect of drug polymer interaction by DSC and surface morphologies by SEM Studies. The *in vitro* dissolution study were analyzed with various kinetic equations like zero order model, first order model, Higuchi model and Korsmeyer-Peppas model in order to understand the mechanism and kinetics of drug release.

Keywords: *Natural mucoadhesive Substance (NMS), pre-gastric absorption, Timolol maleate(TM).*

INTRODUCTION

Timolol maleate(TM) is a beta adrenergic receptor antagonist. In its ophthalmic form it is used to treat open-angle glaucoma and occasionally secondary glaucoma. Since its acceptance in 1979 for ophthalmic applications, Timolol maleate has become the US Drug Administration's 'gold standard' drug for IOP reduction [10]. The bioavailability of ophthalmic drugs is however very poor due to efficient protective mechanisms of the eye. Blinking baseline and reflex, lacrymation and drainage remove rapidly foreign substances including drug from the surface of the eye. More over the anatomy physiology and the barrier function of the cornea comprised the rapid absorption of the drugs [7]. Frequent instillation of eye drops is necessary to maintain a therapeutic drug level in the tear film or at the site of action. But the frequent use of highly concentrated solutions may induce toxic side effects and cellular damage at the ocular surface. Viscous semi-solid preparations such as gels and ointments provide a sustained contact with the eye, but they cause a sticky sensation, blurred vision and induce reflex blinking due to discomfort or even irritation [13]. The mucoadhesive concept which is successful in bucal and oral applications. Some mucoadhesive polymers show good potential to increase the bioavailability of the drug applied is therefore preffered that the composition should be in a form which ensures contact of the active ingredient with the buccal, sublingual, pharyngeal and\or esophageal mucous membrane. Preferably, the composition in the form of microsphere releases the active ingredient in a controllable manner to saliva or to the buccal, pharyngeal and\or esophageal mucous membrane to promote the pre-gastric absorption (U.S.Patent no: 6,297,240). Natural polymers are primarily attractive due to their inherent properties as low cost, easy modification, nontoxic, biocompatible, biodegradable. The mucoadhesive substance used in the present work was obtained from the water soluble extract of the fruit pulp of *D. indica*. According to Kirtikar and Basu [6], the aqueous extract of fruit of *D. indica* contains mostly pectous matter as the polysaccharides. Pectin has been used as a food additive, a thickening agent and a gelling agent [12]. Because of its gelling, film forming and binding properties, biocompatibility, stability

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towards acidic media and non-toxicity, pectin is a very promising biopolymer to construct drug carriers for controlled drug delivery. It would therefore be advantageous to have means for providing an intimate contact of drug delivery system with absorbing membranes[5,] Negai *et al.*, 1984; Ilium *et al.*, 1988).

MATERIALS AND METHODS

Materials

The fruit of *Dillenia indica* was procured from local market and was confirmed by local people,Timolol maleate was a gift from Sun Pharma Ltd.(Vadodora, Gujrat),Sodium Alginate (Loba Chemical Pvt. Ltd., Mumbai, India).All chemicals were of analytical grade laboratory reagents and were used as such.

Extraction and general characterization of mucoadhesive substance

(a) Isolation of mucoadhesive substance

Extraction procedure followed in accordance to that followed for extraction of pectins (Hunda 2003).

(b) Physicochemical Characterization of NMS

The NMS was studied for its various physicochemical properties in terms of its pH, bulk density [4]**,** solubility in various aqueous and non aqueous solvents and effect of various solvents on its swelling [3] and adhesive property.

Preparation of timolol microbeads

The microbeads containing Timolol were prepared by ionotropic gelation method [1]. NMS was added to distilled water and kept as such till it swells completely. Sodium alginate powder was blended with this gel NMS , stirred to form a homogeneous solution. Timolol was added to the above solution and mixed thoroughly to form a smooth viscous dispersion. The dispersions were homogenized at 1500 rpm for 15 minutes at room temperature. The drug polymer dispersions were kept undisturbed for some time to remove air bubbles that might be formed during the stirring process. The dispersion was added drop wise at a constant rate via a 23-gauge needle into a gently agitated solution of cross linking agent. The droplets instantaneously gelled into discrete Timolol loaded beads upon contact with the cross linking agent solution. The microbeads were cured for 10 minutes. Finally the microbeads were hardened by washing in acetone, spread on aluminum foil and air dried for 24 hours, then oven dried at 40°C for 4 hours for the complete removal of water. For optimization of microbeads, the formulation variables like drug concentration, polymer concentration, and concentration of cross linking agent, type of cross linking agents, crcuring time and speed of agitation were used.

Evaluation of prepared microbeads

Particle size measurement

The prepared microbeads were mounted in light liquid paraffin, and the diameters of 100 particles were measured by means of an optical microscope fitted with a stage and an ocular micrometer. The mean diameter was calculated by measuring the number of division of ocular micrometer covering the microbeads [15].The stage micrometer was previously used to standardize the ocular micrometer.

$$\text{Mean particle size} = \Sigma n.d / \Sigma n$$

Drug entrapment Efficiency

The drug entrapment efficiency of beads was estimated by crushing the dried beads and extracting the drug in phosphate buffer (pH 6.8) by vigorous shaking on mechanical shaker for 24 hr. and analyzed the drug content . The entrapment efficiency of microbeads was calculated using the following formula (Wu *et al.,* 2004):

$$\text{Drug entrapment efficiency (\%)} = \frac{\text{Experimental drug content}}{\text{Theoretical drug content}} \times 100$$

Swelling behaviour of microbeads

Swelling behaviour of different batch of microbeads was studied by measuring the percentage water uptake by the microbeads after12hours. About 25 mg of microbeads

Table 1: Formulations with different formulation parameters.

Formulation code / parameter	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11
Timolol maleate(mg)	200	200	200	100	200	200	200	200	200	200	100
Sodium aliginate(mg)	300	300	300	300	300	300	300	300	300	300	200
NMS(mg)	100	150	200	150	150	150	150	150	150	150	150
Cross linking agent(%),$CaCl_2$	8	8	8	8	5	12	_	_	8	8	8
Cross linking agent(%),$BaCl_2$	_	_	_	_	_	_	8	_	_	_	_
Cross linking agent(%),$Al_2(SO_4)_3$	_	_	_	_	_	_	_	8	_	_	_
Curing time(min)	10	10	10	10	10	10	10	10	25	10	10
Stirrring speed(rpm)	200	200	200	200	200	200	200	200	200	400	200

were weighed on electronic balance. Then the microbeads were placed in 20 ml of distilled water, and Phosphate buffer of pH 6.8 and at 37±1^o C after 12hours microbeads were removed from their respective swelling media and weighed after drying the surface water of the microbeads using filter paper. During the procedure, the swollen microbeads were handled carefully in order to avoid any loss due to breaking or erosion of the spheres [1]. The water uptake was calculated in terms of percent water uptake as per formula**.** Percent water uptake= $(W_S \_ W_d)/W_d$ X100, Where Ws = Final weight of microspheres, Wd = Initial weight microspheres.

Table 2: Particle size and Entrapment efficiency of different formulations.

Formulation Code	Mean Particle Size (μm) ± SD	Mean Drug Entrapment Efficiency (%) ± SD
F_1	905.00±14.23	53.23±2.31
F2	934.44±10.58	58.12±1.3
F3	988.12±18.42	65.40±1.35
F4	952.28±18.75	43.31±2.52
F5	1043.00±18.75	48.05±2.54
F6	932.18±11.38	61.47±2.95
F7	971.41±8.98	57.08±2.16
F8	1003.00±16.13	38.05±2.07
F9	982.66±11.14	33.17±2.38
F10	963.40±14.12	35.25±2.07
F11	973.08±17.97	48.15±2.52

Evaluation of the mucoadhesive property

Wash off test

The mucoadhesive property of beads was evaluated by an *in vitro* adhesion testing method known as wash-off method reported[8]. (1990). Freshly excised pieces of goat bucal mucosa (2 × 2cm) were mounted on to glass slides with cyanoacrylate glue. About 50 microbeads were spread on to each prepared glass slide and immediately thereafter the slides were hung to USP tablet disintegration test. When the test apparatus was operated, the sample is subjected to slow up and down movement in the test fluid at 37 ^{0}C contained in a 1-liter vessel of the apparatus. At an interval of 30min up to 10 hours the machine is stopped and number of beads still adhering to mucosal surface was counted. The test was performed in phosphate buffer pH 6.8 media.

Instrumental analysis of the formulation

Scanning Electron Microscopy (SEM)

The surface morphology of the microbeads was studied by using Scanning Electron Microscope (Hitachi, N-3300S, Japan). The microbeads were previously mounted on a brass stub using double-sided adhesive tape and then coated under vacuum (Fine coat , ion sputter , JFC-1100) with a thin layer of gold (3~5nm) for 75 sec and at 40W to make them electrically conductive .The surface morphology of blank microbeads , drug loaded microbeads before and after dissolution were studied by photomicrographs at an excitation voltage of 20 Kv under different magnification.

Drug polymer compatibility study

Differential Scanning Calorimetry (DSC)

To detect any interaction between drug and polymer the DSC thermograms of pure drug, NMS, blank and drug loaded microbeads

were taken using Perkin Elmer JADE DSC System. Blank microbeads and drug loaded microbeads were triturated to get a finely divided powder and the powder was passed through Sieve No.120. Samples (5 -10 mg) were weighted accurately using a single pan electronic microbalance and heated in sealed aluminum pan at a rate of 5^0C/min from 25-500^0C temperature range under a nitrogen flow of 35 ml/min. In the same way, NMS and pure drug were also passed through Sieve no.120 and treated as earlier to record DSC thermograms of individual sample.

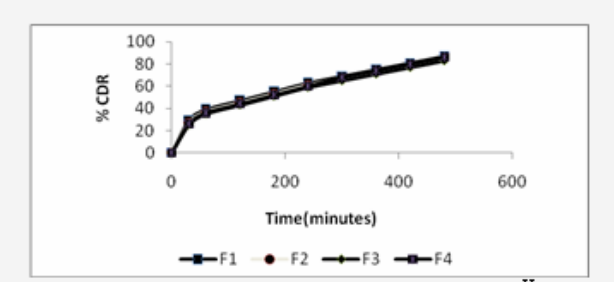

Fig 4.6 (a) Drug release profile in phosphate buffer p^H 6.8.

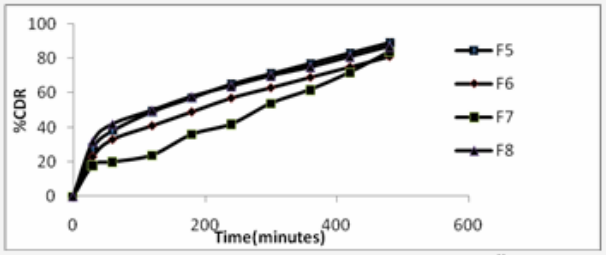

Fig 4.6 (b) Drug release profile in phosphate buffer p ^{H} 6.8.

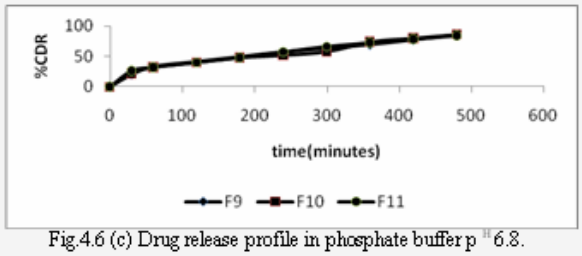

Fig.4.6 (c) Drug release profile in phosphate buffer p ^{H} 6.8.

In *vitro* drug release study

The release of timolol maleate from the microbeads was studied in phosphate buffer P^H 6.8. The tests was performed in IP /BP / USPXXVI dissolution rate test apparatus paddle type (Campbell Electronics , Mumbai) at 37 ± 0.5 °C with a rotating speed of 50 rpm (IP , 1996) . Dissolution test was carried out in triplicate to get reproducible results and the test was carried out 8 hours. A sample of microbeads equivalent to 25 mg of Timolol maleate was used in each test. At preset time intervals 10 ml of sample were withdrawn and replaced by equal volume of fresh dissolution medium kept at the same temperature. The withdrawn samples were filtered through a membrane filter (0.45 µm) and were analysed for drug content spectrophotometrically at 294 nm using UV-Visible Spectrophotometer (Hitachi, model U-2001). Data obtained from in vitro release studies were fitted to various kinetic equations to find out the mechanism of drug release from microbeads. The kinetic models used were Zero order equation, first order equation, Higuchi model and Korsmeyer-Peppas model[2].

RESULTS AND DISCUSSION

Physicochemical characterization of the NMS

From the study it was found that the NMS is insoluble in most of the organic solvents since, it comprises of the acetone insoluble part collected as precipitates during separation. The substance forms colloidal dispersion in water, P.B. and saturated saline and shows very good swelling property in buffer p^H 6.8. The pH of a 2% aqueous solution of NMS was found to be 5.0, which indicates that the extract is composed of acidic molecules. The bulk density of the substance was found to be 0.566g/ml. The NMS showed considerable swelling behavior in water and particularly in phosphate buffer PH 6.8, showing Swelling Index of 590-610% and 680-690% respectively. This may be considered as significant for its use in mucoadhesive drug delivery, particularly for controlled release.

Evaluation of prepared microbeads

Determination of size and size distribution of prepared microbeads

The particle size was determined by optical microscopic method by mounting the microbeads over a glass slide placed on moving stage optical microscope fitted with the calibrated ocular micrometer for analyzing the particle size range. The effect of various parameters on particle size was studied.Morphology of various batches of microbeads containing NMS-sodium alginate was found to be discrete and spherical in shape.

The result indicated that as the amount of NMS in the microbeads was increased, the particle size is also proportionately increased. This could be attributed to an increase in relative viscosity at higher concentration of mucoadhesive polymer results in the formation of larger particles. The mean diameter of the prepared microbeads was marginally increased with an increase in drug loading. The microbead size was comparatively smaller in higher ratio of sodium alginate and also on increasing the concentration of calcium chloride, the particle size was decreased. In both the cases it seems that in higher concentration of the sodium alginate, more MM, GG and MG

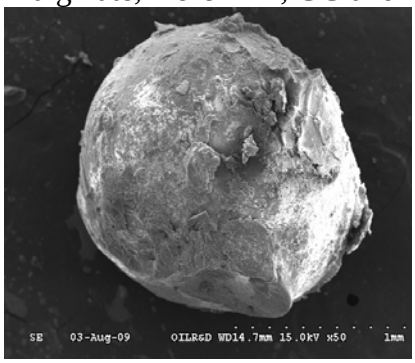

Fig4.3. (a) Scannig electron micrograph of TM loaded NMS microbead at 5x.

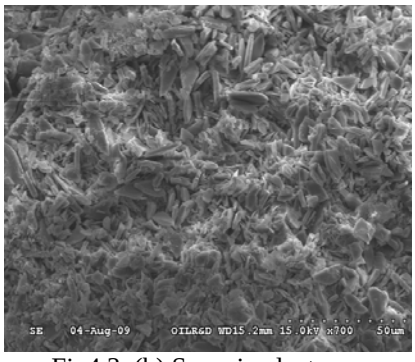

Fig4.3. (b) Scannig electron micrograph of TM loaded NMS microbead at 700X

blocks of alginate are present to be crosslinked. The more crosslinked chains might come in intimate contact occupying small space resulting in smaller particle size. Increase in gel concentration increases the mean particle size of the beads. This is due to the increase in viscosity, which in turn increase the droplet size during addition of the polymer solution to the cross-linking agent solution. Variation in the type of cross linking agents resulted in varying size of microbeads. . Barium Chloride produces smaller micro beads may be due to tight crosslinking between alginate and Ba^{2+}. Aluminium sulphate produces larger microbeads. As a crosslinker calcium chloride is a better option to barium chloride and aluminium sulphate which helps in the formation of larger and spherical particles. The increase in curing time i.e. more time of calcium chloride that is available for cross linking the guluronic acid of sodium alginate results in formation of relatively more cross linked guluronic acid. The increase in curing time reduces particle size. It was found that increase in stirring speed produces small size microbeads compared to that of at low speed due to increased mechanical shearing action of the blades of the stirrer.

Determination of drug entrapment efficiency of microbeads

The drug entrapment efficiency of different formulations have been summarized in the table 4.2. The mean percentage drug entrapment of the formulations was between 33±2.38% to 65.40±1.35%. The Timolol maleate being soluble in water is having tendency to diffuse out to the aqueous medium even though the sufficiently higher drug entrapment to the gel beads prepared with the NMS could be achieved that might be resulted due to hindered diffusion of the medicament through the gel barrier formed by the pectous substances of NMS. The effect of various parameters on percentage drug entrapment efficiency was studied. At higher stirring speed the diffusion of drug from microbeads to the aqueous medium occurs at faster rate and results in low drug load whereas at slow stirring speed microbeads with high drug load were obtained. Increase in stirring time also decreases the drug load due to diffusion of drug into the aqueous medium. Therefore stirring of the crosslinking solution was done at slower rate and the stirring of the crosslinking solution was stopped immediately after the gel dropping completed and the curing of beads was allowed in static liquid medium. A reduction in curing time and increase in the crosslinking agent concentration increases the drug load to microbeads. The drug loading efficiency of the microbeads was also found to be influenced by

the time allowed for homogenization higher homogenization time has shown improved drug entrapment efficiency. The entrapment efficiency was improved in Barium Chloride than Calcium Chloride. It may be due to the tight crosslinking between alginate and Ba^{++} which produces nonporous beads than that of calcium as cross-linking agent. Aluminium sulphate showed a poor entrapping potential.

Mucoadhesion testing

Wash off test

The percentage of microbeads attached to the mucosa after 10 hours has been shown in phosphate buffer (pH 6.8). The microbeads prepared with NMS-Sodium alginate exhibited good mucoadhesive property .It was observed that the beads containing higher percentage of the NMS do not undergo complete wash off in phosphate buffer ph 6.8 rather than the fraction of beads remain on to mucous surface that goes off slowly. It was found that the p^H of the medium is important for the hydration,solubility and mucoadhesion of polymer.It was noted that the NMS fraction of the beads, unlike the sodium alginate that undergoes erosion in phosphate buffer, forms a gel on to the mucous surface that sticks to the mucosal cell surface for a fairly long time. It may be due to receptor–ligand like interaction in which the molecules bind strongly and rapidly directly onto the mucosal surface rather than mucous itself which function with greater specificity.

Moprphological analysis

Surface morphologies of micro beads were investigated with a Scanning Electron Microscope (SEM) at an excitation voltage of 20 Kv under different magnification. The surface morphology of drug loaded microbeads before and after dissolution were studied by photomicrographs. Studies revealed that the NMS containing microbeads were spherical in shape and had a rough surface. Magnification of microbeads to 500 times have shown that the surface contains some crystals deposited in it, which probably was surface associated drug that was required for initial burst release [9]. The photomicrograph taken after 8 hours release study in phosphate buffer P^H 6.8 revealed the development of a porous structure. The surface porosity is crucial for drug release in microbeads prepared with alginate–NMS. Since the polymer is biodegradable, the release of the drug from the microbeads had taken place by diffusion through the pores. The size and number of pores determines the rate and extent of release from the microbeads. Microbeads after dissolution in alkaline buffer medium have become highly porous and fluffy, developed lot of surface cracks which reveals the cause of erosion in the alkaline medium.

Differential Scanning Calorimetry (DSC)

To understand the state of the drug DSC was performed on pure drug, empty microbeads, drug loaded microbeads and NMS. A sharp exothermic peak at about 208^0C was observed for pure Timolol maleate. It was due to an oxidation reaction between Timolol maleate and oxygen in air environment and a melting point of the compound respectively [11]. The DSC thermogram of NMS showed a large decomposition endothermic peak at about 234^0C.It was observed that the large exothermic peak of pure drug was a bit smaller and shifted to 170^0C in drug loaded microbeads revealing its unchanged nature in the microbeads confirming the stability of the drug in the formulation and the large decomposition peak of NMS also shifted to about 165^0 C with decreased intensity in the drug loaded microbeads containing NMS confirming that the polymer is not present in free form where as the DSC thermogram of unloaded microbead showed that there was no sharp peaks. Thus, the consistency of the thermograms of the loaded bead with that of the unloaded bead indicates that the drug has not undergone any chemical interaction with the polymer backbone during the process of microencapsulation [15].

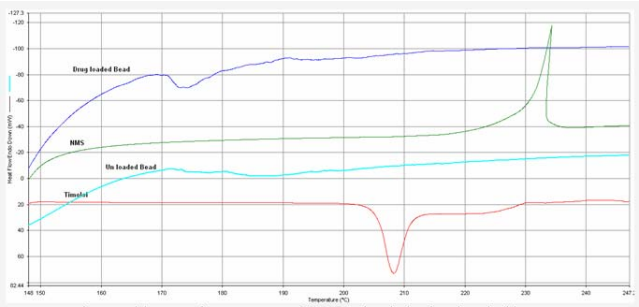

Fig.4.4. (a) DSC thermogram of (a) TM loaded microbead, (b) NMS, (c) Unloaded microbead, (d) Timolol maleate

***In vitro* drug release study**

The *in vitro* dissolution profiles were carried out in the dissolution media phosphate buffer p^H 6.8 to observe the pre–gastric absorption (prior to the stomach i.e buccal, sublingual and esophageal absorption) as the p^H of the bucal cavity is 6.8. It was observed that the alkaline pH caused greater stress to the polymer backbone causing release of medicament. The beads were swelled excessively followed by the erosion in buffered alkaline medium. The NMS fraction of the beads shown the gel forming ability in alkaline medium this can be attributed to be beneficial for sustaining drug release by forming the gel barrier. The sustained release for sufficient long duration was obtained from the micro beads containing NMS that appears to be resulted due to hindered diffusion from the gel matrix formed *in situ* by the NMS and there by reduced drug release.

Kinetic modeling of drug release profiles

In order to understand the mechanism and kinetics of drug release data of the *in vitro* dissolution study were analyzed with various kinetic equations like zero order model (% cummulative drug release Vs time), first order model (log % amount remaining Vs time), Higuchi model (% cummulative drug release Vs Square root of time) and .Korsmeyer- Peppas model (log % cummulative drug release Vs log time). Coefficient of correlation (R^2) values were calculated for the linear curves obtained by regression analysis of the above plots. The release profile followed all the kinetic models Zero order, First order and Higuchi model. The obtained correlation coefficient values indicated that the drug release followed the diffusion control mechanism from the microbeads. The data's are supportive to the findings that a drug incorporated in the swellable matrix device is mainly released by diffusional mechanism [14]. The data obtained was also put in Korsemeyer-Peppas model in order to find out 'n' value, which describes the drug release mechanism. The 'n' value of formulations of different polymer to drug ratio indicates that the mechanism of the drug release to be diffusion controlled. The release also showed high correlation with Korsmeyer-Peppas model. The 'n' values of all formulations were between 0.5-1 as such it shows an anomalous type of release which is influenced by both diffusion of drug and swelling of the gel matrix (NMS).

CONCLUSION

It was found that NMS along with sodium alginate substantially sustained and modulated in controlling the release of TM from the microbeads. Results of the in vitro drug release indicated that the controlled drug release upto 8 hours were obtained from the so prepared carrier backbone. These studies demonstrated that TM can be encapsulated into microbeads having NMS and sodium alginate backbone by ionic gelation technique having good batch to batch reproducibility with respect to yield, particle size, entrapment efficiency and in vitro drug release profile of micro beads. In conclusion the performed studies suggested that the NMS may be a promising candidate for oral controlled drug delivery system because of its gel forming ability and sustaining the release of drug. The microbeads also showed considerable swelling behavior in phosphate buffer pH 6.8, which is the favourable condition for pre-gastric absorption. The prepared microbeads showed controlled drug delivery behavior as can be inferred from the release kinetics data, as it follows zero order, first order as well as Higuchi model which confirms its diffusion controlled release behavior although the Korsemeyer-Peppas plot shows anamalous transport suggests that the release is controlled by diffusion as well as by other factors like swelling of the microbeads.

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