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Study On Effect of Formulation Variables on Drug Release of Bosentan Microspheres

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The scope of the study was to examine the effect of stirring speed and polymer ratio on characteristics of Bosentan (BOS) microspheres. In the present study BOS microsphere formulations were prepared by ionotropic gelation process using Sodium alginate, and polymers Hydroxypropyl methyl cellulose (HPMC) K15 and Eudragit RL 100, either alone or in combination at different compositions. A total of twenty-four formulations were developed, with twelve prepared at 400 rpm and twelve at 600 rpm. The effects of drug-polymer ratio and stirring speed on practical yield, particle size, entrapment efficiency (EE), and in vitro drug release were investigated. The practical yield of formulations prepared at 600 rpm ranged from 82.6 ± 1.15% to 92.3 ± 1.37%, while at 400 rpm, it ranged from 80.2 ± 1.62% to 90.1 ± 1.26%. The particle size of formulations prepared at 600 rpm ranged between 167.2 ± 1.34 μm and 192.8 ± 1.021 μm, whereas at 400 rpm, it was found to be between 186.9 ± 1.03 μm and 204.9 ± 1.05 μm. The entrapment efficiency (EE) for F1–F12 at 600 rpm was 72.5 ± 1.37% to 84.1 ± 0.92%, while at 400 rpm, it ranged from 79.8 ± 0.25% to 90.8 ± 0.42%. Among all the formulations, F6, prepared at 600 rpm, was identified as the optimized formulation as it had an optimal particle size of 172.9 ± 1.05 μm and an EE of 84.1 ± 0.92%. It also achieved 100% drug release within 12 hours in a controlled and predetermined manner. The results indicated that the combination of HPMC K15 and Eudragit RL 100 at optimal concentrations, along with higher stirring speeds, significantly enhanced drug release characteristics.

Keywords: Bosentan; Eudragit RL 100; HPMC K15; Ionotropic gelation; In vitro; Sodium alginate.

A well-known technique to change and delay properties of drug release is the use of microspheres. Microspheres are used to sustain and control drug release when administered orally. Compared to conventional dosages, the administration of drugs using microspheres promotes uniform distribution, leading to consistent absorption and reduced localized discomfort [1].

Microspheres produce controlled drug delivery, and they provide different structural modifications for altered drug release. Due to their advantages in target specificity and improved patient compliance, microspheres are considered a superior option for drug delivery systems. Conventional formulations have several drawbacks as they require multiple doses Studies show that maintaining a constant plasma concentration through controlled-release dosage forms enhances therapeutic efficacy [2]. This steady release makes microspheres an attractive solution for prolonged and controlled drug administration.

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The drug's yield, morphology, release, and entrapment within the system are influenced by various factors, including the type of polymer, its concentration, the drug-to-polymer ratio, stirring speed, and concentration of cross-linking agents. Therefore, these elements are essential to the design of microspheres.

The main objective of the study was to evaluate the influence of polymer concentration, drug-to-polymer ratio, stirring speed on particle size, entrapment efficiency (EE), and *in vitro* release profile of microspheres, with the goal of optimizing the formulation for improved therapeutic efficacy and stability.

Bosentan (BOS), which is used to treat pulmonary arterial hypertension, was selected as a model drug for microsphere preparation due to its poor solubility in aqueous media which necessitates frequent dosing, and increases the cost of therapy [3]. Microspheres can enhance the drug's bioavailability, thereby reducing the frequency of administration, improving patient compliance, and ensuring controlled drug release.

MATERIALS AND METHODS

Materials

BOS was a gift sample from SD Fine Chemicals, Mumbai. Sodium alginate (CDH, New Delhi), Calcium chloride ($CaCl_2$) (Qualigens, Mumbai), Eudragit RL100 (SD Fine Chemicals, Mumbai), Hydroxypropyl methyl cellulose (HPMC K15), Hexane (Spectrochem Pvt. Ltd Mumbai) were commercially procured. All other reagents used in the experiment were of analytical grade and purchased from commercial sources.

Method

Formulation of BOS Microspheres

Twenty-four formulations of BOS microspheres were prepared by the ionotropic gelation process using Sodium alginate, along with two polymers, HPMC K15 and Eudragit RL 100, and $CaCl_2$, with twelve formulations at a stirring speed of 400 rpm and twelve at 600 rpm. HPMC K15 and Eudragit RL100 were used at different concentrations alone, as well as in combination. Three sets of BOS microspheres were prepared: Alginate-HPMC-Eudragit, Alginate-HPMC, and Alginate-Eudragit. The composition of these formulations is presented in Table 1. The preparation involved three steps. First, preparation of a homogeneous solution of 1% Sodium alginate in distilled water by continuous stirring on a magnetic stirrer. HPMC K15 and Eudragit RL100 were then added separately according to the formulation details in Table 1. Second, a drug solution was prepared in dichloromethane and gradually added to the polymer solution under stirring. Third, a 5% $CaCl_2$, solution was prepared, with one beaker set at stirring speed 400 rpm and another at 600 rpm. The drug-polymer solution was then added dropwise into the $CaCl_2$, solution using a syringe with a 20-gauge needle. The formed microspheres were filtered, washed multiple times with hexane and water, and dried in a hot air oven at 50°C. Other parameters, such as the recommended dosage (62.5 mg) and the volume of the 5% $CaCl_2$, solution, remained unchanged.

Compatibility studies

The Potassium bromide (KBr) pellet approach allows for high-quality acquisition of the IR spectrum. Pellets were prepared from pure drug powder and from a mixture of the drug and excipient (F6 microspheres) along with KBr. These pellets were then exposed to an infrared beam, and spectra were recorded using Bruker Alpha with Opus software.

Evaluation of BOS Microspheres

Practical Yield of BOS Microspheres

Microspheres that had been thoroughly dried were precisely weighed and the % yield was determined using following formula [4].

% Yield = mass of microsphere obtained / total weight of drug and polymer X 100

Particle Size Analysis

Standard sieving technique was used for particle size distribution analysis by screening microspheres for 10 minutes in a mechanical shaker with conventional sieves having I.P.-compliant pore sizes. Microspheres were divided into various size fractions, and the particle size distribution was identified [5,6]. The mean particle size of the microspheres was determined by using the following formula:

Mean particle size = (mean particle size of fraction X weight fraction) / weight fraction

Particle Surface Morphology

Particle surface morphology of optimized formulation was determined usingscanning electron microscope (SEM) to study the shape and surface of microspheres [7].

BOS microspheres Entrapment Efficiency [EE]

Precisely weighted 10 mg microspheres were broken up and mixed with 25 ml of pH 6.8 phosphate buffer in order to assess EE. The prepared mixture was shaken for 24 hrs. The solution was filtered after 24 hrs, and the filtrate was subjected to an appropriate dilution before being examined at 271 nm with a UV spectrophotometer to determine drug content [8].

***In vitro* Drug Release of BOS Microspheres**

Microspheres were subjected to 12hr *in vitro* drug release test utilizing a USP rotating apparatus set at 50 rpm and a temperature of 37±0.5°*C.* An *in vitro* drug release investigation was conducted in 900 ml of 1.2 pH, 0.1 N HCl for the first 2 hrs., then in 900 ml of pH 6.8 phosphate buffer for 3 to 12 hrs. At time intervals of 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 hrs, 5 ml of dissolving medium containing BOS was withdrawn, and the volume was replaced with an equal amount of fresh medium to maintain the total volume constant. Samples were filtered to remove any particulate matter, ensuring that only dissolved BOS was measured. Filtered samples were then analyzed using a UV spectrophotometer, and the absorbance of BOS at 271 nm was measured, which correlates with its concentration in the medium [8,9].

Drug Release Kinetics of BOS Microspheres

The mechanism and kinetics of drug release from the developed microsphere formulations were determined by fitting the data obtained from the *in vitro* dissolution study into zero-order, first-order, Higuchi, and Korsmeyer-Peppas release models [9].

Stability Studies of BOS Microspheres

The purpose of stability testing is to provide evidence on how the quality of a drug product varies with time under the influence of various environmental factors such as temperature, humidity, and light. The optimized BOS microsphere formulation was filled in tightly closed glass vials and subjected to stability testing according to the International Conference on Harmonization (ICH) guidelines. Microspheres were kept at refrigerated temperature (4 ± 2ÚC) and at room temperature (25 ± 2ÚC) in a stability chamber for 3 months and were evaluated for particle size, drug content, and entrapment efficiency. All the determinations were conducted in triplicate [10].

RESULTS AND DISCUSSION

Compatibility studies

The analysis of the infrared spectrum of the pure drug (Figure 1) and optimized formulation F6 (600 rpm) (Figure 2) showed the presence of similar characteristic bands of BOS in the Bosentan-loaded microspheres, indicating no chemical interaction with the polymers.

Table 1. Composition of different formulations of BOS microspheres

Formulation	Drug (mg)	Sodium alginate (mg)	HPMC (mg)	Eudragit (mg)	$CaCl_2$ (%)
F1	62.5	1000	125	125	5
F2	62.5	1000	250	250	5
F3	62.5	1000	375	375	5
F4	62.5	1000	125	250	5
F5	62.5	1000	250	375	5
F6	62.5	1000	375	125	5
F7	62.5	1000	125	-	5
F8	62.5	1000	250	-	5
F9	62.5	1000	375	-	5
F10	62.5	1000	-	125	5
F11	62.5	1000	-	250	5
F12	62.5	1000	-	375	5

Practical Yield of BOS Microspheres

The practical yield of formulations prepared at 600rpm ranged from 82.6 ± 1.15% to 92.3 ± 1.37% and while those prepared at 400 rpm ranged from 80.2 ± 1.62% to 90.1 ± 1.26%. The data demonstrate that the practical yield of microspheres prepared with single polymer -HPMC in formulations F7–F9 and Eudragit

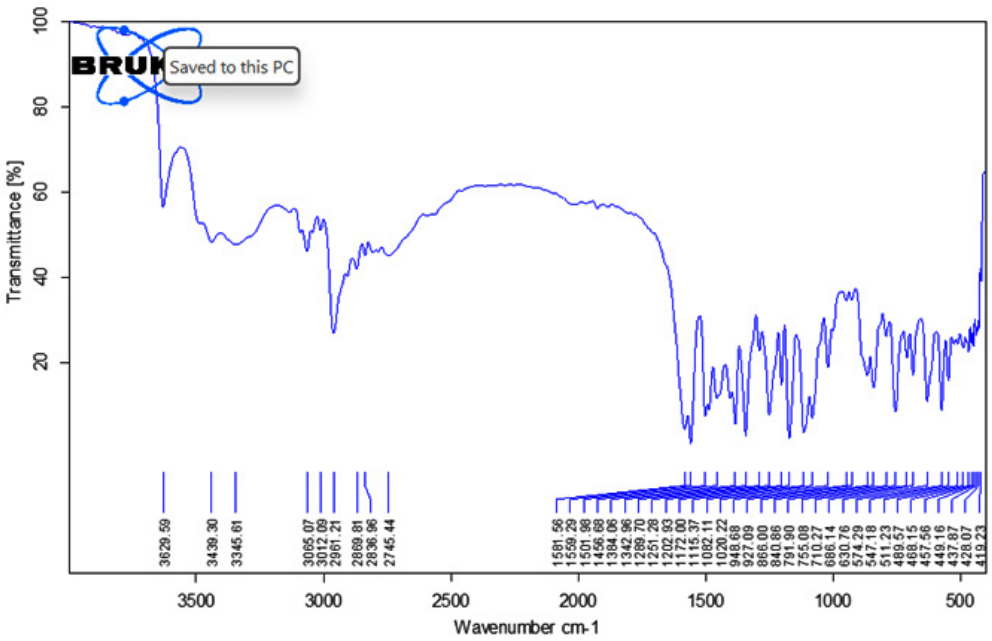

Fig. 1. FTIR spectra of BOS pure drug

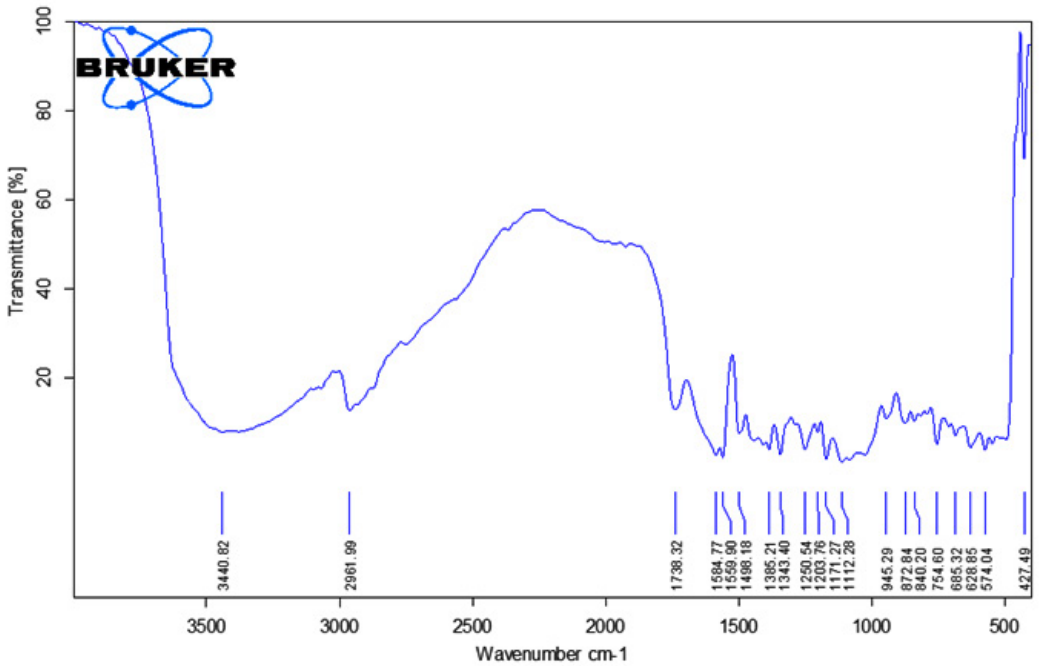

Fig. 2. FTIR spectra of optimized formulation of BOS microspheres

in F10–F12 followed a consistent pattern. The practical yield decreased as the concentration of HPMC or Eudragit increased in the respective formulations. This decrease in practical yield can be attributed to increased solution viscosity at higher polymer concentrations, which reduces syringeability due to needle blockages, leading to wastage of the drug-polymer solution. High viscosity also promotes agglomeration and causes the solution to adhere to stirrers and beakers, further reducing yield [11, 12, 13]. However, the remaining formulations, F1-F6, prepared using a combination of both polymers, exhibited inconsistent trends in practical yield. This suggests that interactions between the two polymers may influence the yield in a less predictable manner. Despite having a high polymer content, F3 exhibited a low practical yield. When the practical yield was compared at different speeds 400 rpm and 600 rpm, formulations at lower speeds showed reduced yields. Lower stirring speeds result in insufficient mixing, leading to non-uniform dispersion, particle agglomeration, and inefficient droplet breakup. Consequently, this produces larger particle sizes and lower yields [14]. The results are summarized in Table. 2.

Particle Size Analysis of BOS Microspheres

The particle size of the microspheres was influenced by the polymer ratio and stirring speed. The particle size of formulations F1- F12 prepared at 600 rpm, ranged between 167.2 ± 1.34 to 192.8 ±

Table 2. Practical yield and Particle size of BOS microsphere formulations at different stirring speeds

Formulation	Practical yield (%)		Particle size (µm)	
	At 600 rpm	At 400 rpm	At 600 rpm	At 400 rpm
F1	89.2 ± 1.02	86.9 ± 1.01	171.2 ± 1.34	198.1 ± 1.04
F2	88.5 ± 1.25	85.7 ± 1.05	175.1 ± 1.19	199.7 ± 1.11
F3	82.6 ± 1.15	80.2 ± 1.62	192.8 ± 1.02	204.9± 1.05
F4	89.1 ± 1.38	87.1 ± 1.28	178.5 ± 1.25	201.9 ± 1.12
F5	87.2 ± 1.41	85.2 ± 1.41	183.3 ± 1.37	193.9 ± 1.20
F6	89.2 ± 1.24	87.9 ± 1.24	172.9 ± 1.05	192.8 ± 1.50
F7	92.3 ± 1.37	90.1 ± 1.26	171.2 ± 0.96	188.9 ± 0.99
F8	89.5 ± 1.49	87.8 ± 1.37	174.5 ± 1.21	199.5 ± 1.02
F9	89.2 ± 1.17	85.9 ± 1.17	189.1 ± 1.06	200.6 ± 1.01
F10	91.4 ± 1.04	89.9 ± 1.04	167.2 ± 1.34	186.9 ± 1.03
F11	90.1 ± 1.02	87.8 ± 1.62	168.7 ± 1.27	193.6 ± 1.06
F12	89.7 ± 1.14	86.2 ± 1.14	172.4 ± 1.02	198.9 ± 1.09

All values are expressed in mean ± SD, (n=3)

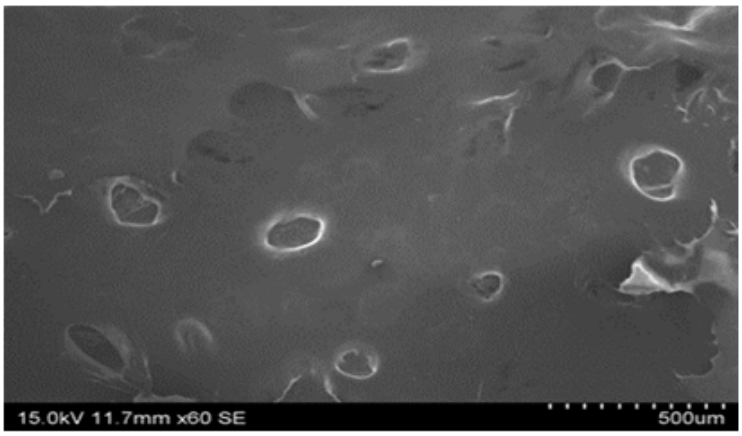

Fig. 3. SEM images of microspheres of optimized formulation F6 at 600 rpm

1.021 µm, while those prepared at 400 rpm ranged from 186.9 ± 1.03 to 204.9 ± 1.05 µm, all within the acceptable limits.

In formulations containing single polymer HPMC in F7-F9 and Eudragit in F10-F12, the particle size increased as the polymer concentration increased. Formulations containing both polymers F1-F6, prepared at stirring speed 600 rpm, had smaller particle size compared to those prepared at 400 rpm. The mean size of the microspheres decreased as the stirring speed increased. Increased agitation force distributes the internal phase into smaller droplets, resulting in the formation of microspheres with smaller particle sizes. In formulations containing a single polymer from F7-F12, the particle size was larger at 400 rpm than at 600 rpm stirring speed due to the lower shear force and particle agglomeration [14, 15, 16]. The results are shown in Table 2.

Table 3. Comparative study for EE of BOS microspheres

Formulation	Drug EE (%)	
	At 600 rpm	At 400 rpm
F1	75.2 ± 0.95	79.8 ± 0.25
F2	78.6 ± 1.36	87.4± 1.06
F3	81.3 ± 1.05	89.3 ± 1.09
F4	75.9 ± 1.14	88.9 ± 1.04
F5	79.4 ± 0.89	89.3 ± 0.81
F6	84.1 ± 0.92	90.8 ± 0.42
F7	74.0 ± 1.09	85.5 ± 1.12
F8	77.7 ± 1.25	86.7 ± 1.25
F9	79.8 ± 0.98	88.8 ± 0.67
F10	72.5 ± 1.37	83.9 ± 0.97
F11	74.3 ± 1.13	89.7 ± 1.03
F12	76.2 ± 1.24	89.9 ± 1.24

All the values are expressed in mean ± SD, (n=3)

Surface Morphological Studies

Optimized formulation F6 microspheres were spherical and had a smooth surface without pores, and pale yellow in color. The SEM image is shown in Figure 3.

Entrapment Efficiency of BOS Microspheres

The entrapment efficiency (EE) of formulations F1-F12 prepared at stirring speeds 600 rpm and 400 rpm, ranged from 72.5 ± 1.37 to 84.1 ± 0.92% and 79.8 ± 0.25 to 90.8 ± 0.42%, respectively. These results demonstrate the influence of both stirring speed and polymer concentration on EE.

EE was higher at a stirring speed of 400 rpm compared to 600 rpm. At lower stirring speeds (400 rpm), gentler mixing allows the drug and

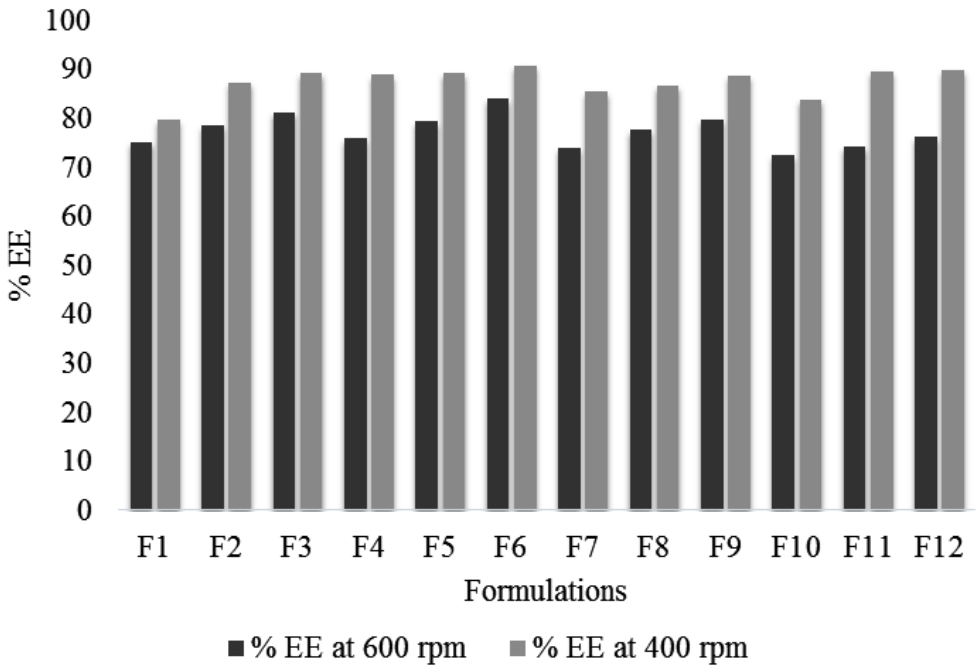

Fig. 4. Comparison of % EE of BOS microspheres at different stirring speeds

polymer to interact more uniformly, promoting the formation of stable and larger droplets or particles. This leads to better encapsulation of the drug within the polymer matrix. In contrast at higher stirring speeds (600 rpm), increased shear forces can disrupt the droplet stability during formulation, resulting in incomplete drug encapsulation or drug leakage, which lowers EE [15, 16]. Formulations

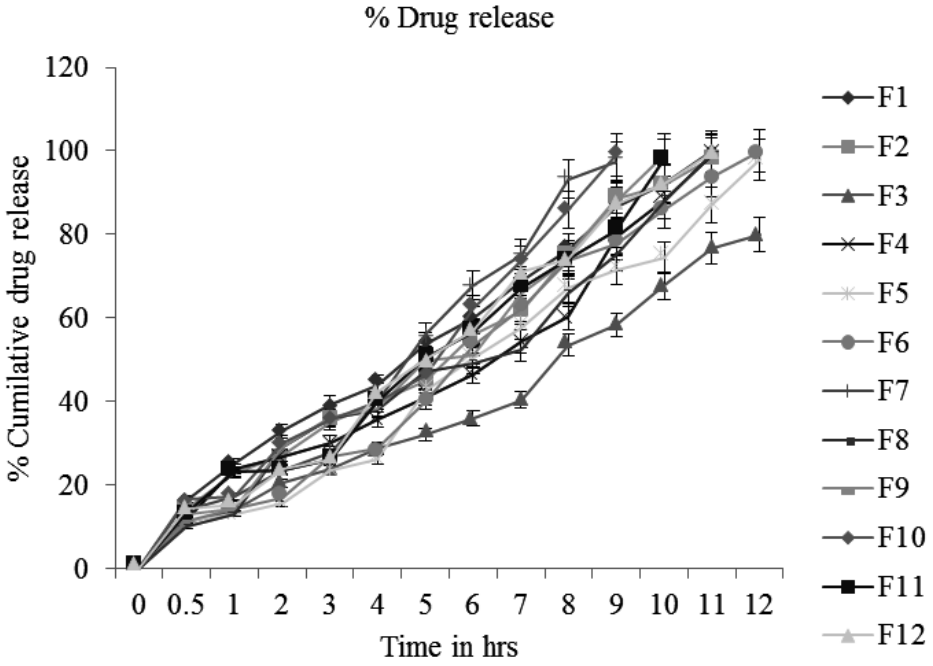

Fig. 5. *In vitro* drug release profile of BOS microspheres (n=3) at 600 rpm

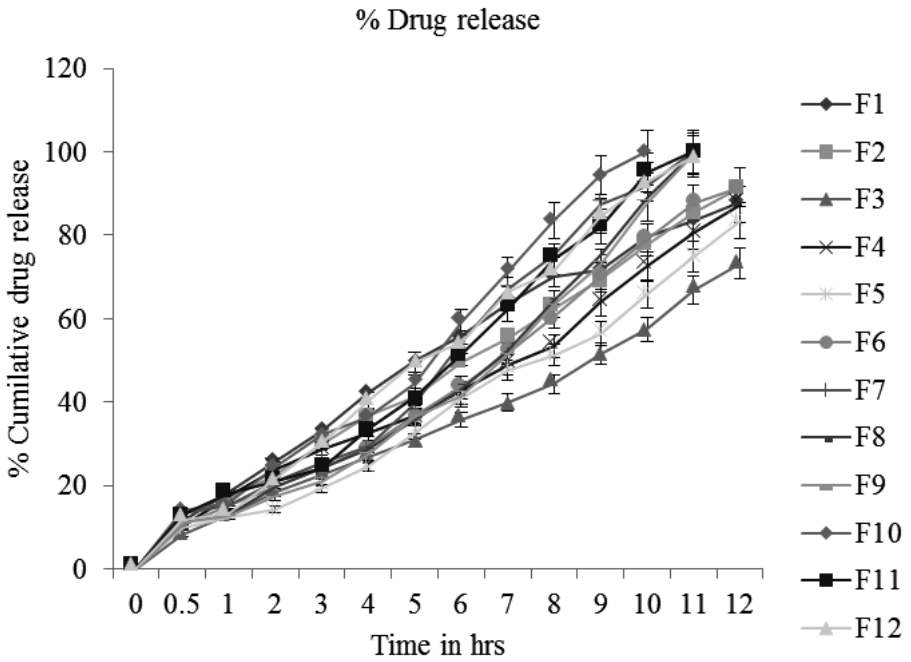

Fig. 6. *In vitro* drug release profile of BOS microspheres (n=3) at 400 rpm

Table 4. Drug release kinetics of BOS microspheres prepared at 600 rpm

Formulation	Zero-order	First order	Higuchi model	Korsmeyer Peppas
F1	0.989	0.733	0.991	0.979
F2	0.983	0.788	0.974	0.971
F3	0.969	0.901	0.964	0.962
F4	0.985	0.637	0.965	0.953
F5	0.985	0.734	0.960	0.864
F6	0.996	0.884	0.872	0.858
F7	0.995	0.809	0.957	0.969
F8	0.989	0.847	0.970	0.971
F9	0.991	0.859	0.970	0.973
F10	0.989	0.862	0.966	0.967
F11	0.967	0.693	0.971	0.967
F12	0.994	0.696	0.973	0.980

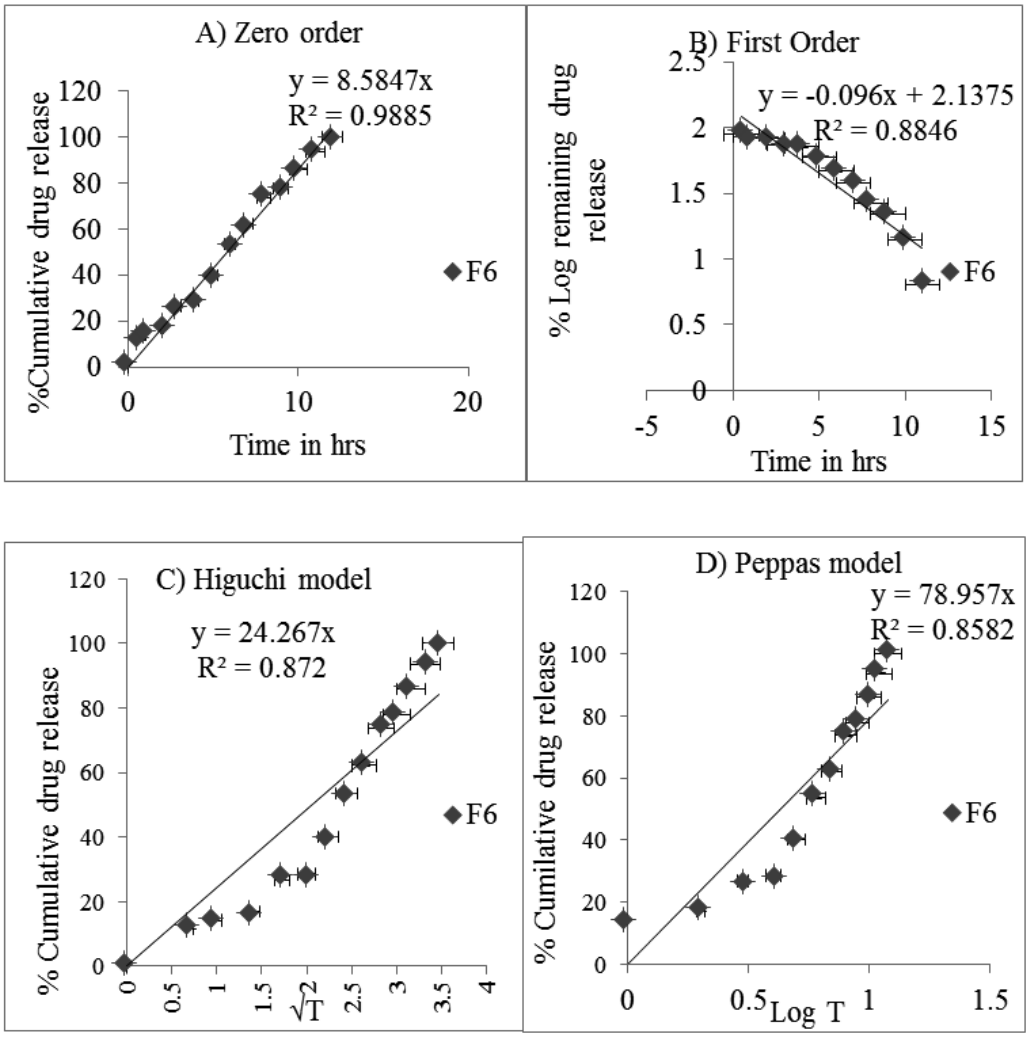

Fig. 7. Drug release kinetics of optimized formulation F6 A) First order, B) Zero order, C) Higuchi and D) Peppas model

Table 5. Stability studies of optimized formulation F6 (600 rpm) at Room temperature and Refrigerated temperature

Storage condition	Time intervals (Months)	Particle size (μm)	EE (%)
Room temperature	Initial	172.9 ± 1.05	84.1 ± 0.92
(25 ± 2°C)	1	172.5 ± 1.02	84.1 ± 0.37
	2	172.3 ± 1.19	84.1 ± 0.14
	3	172.1± 1.19	84.1 ± 0.12
	6	171.8 ± 1.21	84.1 ± 0.05
Refrigerated temperature	Initial	172.9 ± 1.05	84.1 ± 0.92
(4 ± 2°C)	1	173.3 ± 1.35	84.1 ± 0.87
	2	173.5 ± 1.14	84.1 ± 0.79
	3	173.7 ± 1.24	84.1 ± 0.77
	6	174.1 ± 1.27	84.1 ± 0.52

All values are expressed in mean ± SD, (n=3)

containing a single polymer, HPMC in F7-F9 and Eudragit in F10-F12, showed a trend of increasing EE with increasing polymer concentration. This can be attributed to the greater availability of polymer molecules at higher concentrations, which enhances drug encapsulation. The cohesive and adhesive properties of HPMC and the film-forming ability of Eudragit contribute to the improved EE as their concentration increases. However in formulations containing both HPMC and Eudragit, the EE appeared inconsistent. This could be due to the incompatibility or competitive interactions between the two polymers, which might hinder the uniform drug encapsulation. Differences in their viscosities, solubilities, or interaction mechanisms could lead to uneven drug distribution or encapsulation efficiency.

The results indicate that the EE is significantly influenced by stirring speed and polymer concentration. Lower stirring speeds (400 rpm) promote higher EE due to gentler mixing, while higher polymer concentrations enhance encapsulation capacity. However, compatibility and interactions between different polymers (HPMC and Eudragit) play a crucial role, and their inconsistent behaviour highlights the need for optimization in such systems [17, 18, 19]. The results are presented in Table 3 and Figure 4. Several other factors also affect the EE of microspheres. One of such factors is the presence of Calcium chloride cross-linking ions in the gelation fluid for alginate, which influences ionotropic gelation. Longer gelation durations can lead to increased drug loss and diffusion. Additionally, the size of polymer-drug droplets impacts EE, as smaller droplets have a larger surface area, making drug loss more likely. Achieving high EE in ionotropic gelation requires optimizing two interdependent factors; polymer type and mixing speed. While high-viscosity polymers and low mixing speeds enhance drug retention, moderate conditions often provide the best balance between stability, homogeneity, and efficiency. Experimental optimization, including adjustments to polymer concentration and stirring rates, is crucial for improving EE [17].

***In Vitro* Dissolution Studies of BOS Microspheres**

Release studies for all the formulations conducted over a 12-hour period provided significant insights into the effect of stirring speed, polymer concentration, and polymer combinations on drug release behaviour.

In formulations containing a combination of polymers (F1–F6) prepared at 600 rpm, drug release varied depending on the polymer ratio. F1 released 99.9% of the drug in 11 hours, while F2 released 98.3%. F4 released 98.9% in 11 hours, and F5 released 98.1% within 12 hours. F6 demonstrated controlled release, achieving 100% drug release within 12 hours, making it the most suitable formulation for sustained drug release. F3, which had a high polymer combination ratio, exhibited an extended-release profile of 80% over 12 hours, demonstrating the retarding effect of high polymer content on drug diffusion. In single-

polymer formulations (F7–F12), increasing the concentration of HPMC K15 in F7–F9 and Eudragit RL 100 in F10–F12 failed to extend drug release to 12 hours. This highlights the limitations of single polymers and underscores the need for synergistic polymer combinations. Among all formulations, F6 emerged as the optimal formulation, achieving a perfect balance between drug release rate and duration, ensuring complete (100%) drug release over 12 hours. This performance reflects the effectiveness of the specific combination and ratio of HPMC K15 and Eudragit RL 100 in F6 [16, 17, 20.] The drug release profiles for microsphere formulations prepared at 600 rpm are shown in Figure 5.

Drug release for microspheres prepared at 400 rpm were conducted under conditions comparable to those at 600 rpm. Across all formulations, drug release decreased compared to their 600 rpm counterparts. This reduction can be attributed to the lower surface area and larger particle size of the microspheres formed at the lower stirring speed, which likely resulted in a decreased drug diffusion rate. None of the formulations prepared with a combination of polymers achieved 100% drug release within 12 hours at 400 rpm, further emphasizing the role of stirring speed in determining release characteristics. Unlike the combination-polymer formulations, single-polymer formulations (F7–F12) achieved 100% drug release within 11 hours, indicating that stirring speed influenced release profiles differently for single-polymer and combination-polymer systems. Among all formulations prepared at both 400 rpm and 600 rpm, F6 at 600 rpm remained the most effective, releasing 100% of the drug over 12 hours with controlled release characteristics. Compared to previous studies on BOS microcapsules with high concentrations, the present study, using a lower concentration, exhibited sustained release for up to 12 hours and extended the release, following zero-order kinetics[21]. The results are illustrated in Figure 6.

Drug Release Kinetics of BOS Microspheres

The drug release kinetics of microsphere formulations prepared at 600 rpm provide critical insights into their behavior and optimization. All formulations exhibited a linear release profile with zero-order kinetics, with R^2 values ranging from 0.967 to 0.996. Zero-order kinetics indicate that drug release was independent of concentration, ensuring a constant release rate over time. The microsphere formulations also demonstrated linearity with first-order kinetics, with R^2 values between 0.637 to 0.901. First-order kinetics suggests that drug release was concentration-dependent; however the weaker correlation compared to zero-order kinetics implies that zero-order behaviour predominates. Additionally, release profiles aligned with the Higuchi model, with R^2 values ranging from 0.872 to 0.991, indicating that drug release was influenced by diffusion through the polymeric matrix. The release exponent (5Ø[Ü) values obtained from the Peppas model ranged from 0.858 to 0.980, suggesting a non-Fickian diffusion mechanism. Non-Fickian (anomalous) diffusion implies that drug release was governed by a combination of diffusion and polymer erosion processes. Based on the R^2 values and the release mechanisms, it was concluded that all formulations predominantly followed zero-order kinetics. This behavior ensures controlled and predictable drug release at a predetermined rate, making these formulations suitable for sustained drug delivery [18]. The results are given in Table 4, and drug release kinetics of optimized formulation F6 illustrated in Figure 7.

Stability Studies of Optimized BOS Microspheres

Stability studies for formulation F6, prepared at 600 rpm, confirm its robustness under different storage conditions. A slight decrease in particle size was observed, possibly caused by the evaporation of residual organic solvent from the microspheres, driven by higher ambient temperatures. A slight increase in particle size was noted, likely due to the aggregation of microspheres caused by condensation or reduced mobility at low temperatures. The minor variations in particle size are within acceptable limits and do not impact the formulation's performance. No significant changes in EE and drug content were observed under both room and refrigerated conditions. This indicates that the formulation retained its drug-loading capacity and chemical integrity, showing resistance to degradation or loss of efficacy over time. The results demonstrate that formulation F6 remains stable under both room and refrigerated storage conditions and support the practical usability of F6 for sustained drug delivery applications [22]. Table 5 presents the detailed results of the stability study.

CONCLUSION

The present study successfully developed BOS microspheres using ionotropic gelation, offering an effective strategy for sustained drug release. By optimizing the drug-polymer ratio and stirring speed, the formulations achieved zero-order release kinetics, ensuring a constant and controlled release of the drug over a 12-hour period. Among the various formulations, F6 (600 rpm) demonstrated superior performance, exhibiting high entrapment efficiency, minimal burst release, and complete drug release, making it the most promising formulation for long-term drug delivery**.** These findings highlight how careful control of formulation parameters can significantly impact the efficiency and effectiveness of microsphere-based drug delivery systems, offering a more reliable treatment option.

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Conflict of interest

The authors do not have any conflict of interest.

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials**.**

Author Contributions

Conceptualization, Ch. Praveena, M. Anitha: Writing – original draft preparation; M. Anitha: Writing – original draft preparation; Ch. Praveena: writing review and editing.

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