FORMULATION AND EVALUATION OF DIAZEPAM-LOADED INTRAVAGINAL ALGINATE BEADS: AN INVESTIGATION STUDY FOR THE TREATMENT OF PELVIC FLOOR DYSFUNCTION

SHEREEN AHMED SABRY*
Department of Pharmaceutics and Industrial Pharmacy, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt.
Email: Shereensabry134@yahoo.com
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ABSTRACT

Objective: The fundamental objective of this research investigation was to develop intravaginal diazepam (DZ)-loaded alginate beads for the management of acute pelvic floor dysfunciton (PFD) pain with minimal sedative effect.

Methods: DZ loaded beads were prepared by an ionotropic gelation method using SA (sodium alginate) alone, or in combination with either poloxamer 407 (PL), pectin (PC), or xanthan gum (XG) at different ratios in the presence of different concentrations of calcium chloride as a cross-linking agent. The successfully developed beads were evaluated for the particle size, pH, yield percentage, entrapment efficiency, in vitro bioadhesion, swelling percentage, and in vitro drug release. The stability, ex vivo drug permeation, and sedative action of the optimized beads formulations were studied.

Results: The particle size of the formulated beads was from 395±3.3 to 515±2.8 µm, yield percentage was from 68.2±1.7 to 87.5±2.1, entrapment efficiency was from 65.6±1.6 to 87.5±2.1, pH ranged from 6.1±0.2 to 6.8±0.6, bioadhesion strength was from 71.5±1.3 to 87.6±3.1, and swelling percentage was in the range from 53.4±3.1 to 85.2±3.7. Approximately 92.4–72.6% of the loaded dose was released from the prepared beads. The optimized beads showed a good stability under the selected storage conditions. About 74.8%, 71.1%, 68.6%, and 63.4% of the loaded dose permeated through the rabbit vaginal mucosa from F7, F9, F3, and F11, respectively. The formulated beads decreased the sedative action associated with orally or parenterally administered DZ.

Conclusion: The developed beads were considered a promising candidate to formulate DZ into a new dosage form for the treatment of PFD with a minimum central nervous system sedation.

Keywords: Diazepam, Intravaginal alginate beads, Ionotropic gelation, Pelvic floor dysfunction.

INTRODUCTION

The vaginal drug delivery system is currently of great interest in producing local and systemic action [1,2]. The importance of the vagina as a route of drug administration comes from its rich blood supply, large surface area, high permeability to several drugs, in addition to the avoidance of the first-pass metabolism [3]. Mucoadhesion has been of great interest in the development of vaginal drug delivery systems to increase the residence time at the site of absorption [4]. Sharma et al. formulated alginate beads loaded with voglibose for the effective treatment of hyperglycemia [5]. It was reported that glipizide-loaded alginate-chitosan microspheres were an effective approach for the treatment of diabetes [6]. Abdelatif et al. formulated flurbiprofen into calcium alginate beads to mask the drug taste and its burning effect in the oral cavity and stomach and to improve its poor bioavailability due to extensive first-pass metabolism [7]. Bioadhesive polymers such as SA, XG, PC, and PL exhibited a good stability in a wide range of pH and were considered good candidates for the vaginal drug delivery [8]. Pelvic floor dysfunction (PFD) is a collective term for a variety of disorders that occur when pelvic floor muscles and ligaments are impaired. Sexual pain, pelvic pain, pressure, incontinence, incomplete emptying, and visible organ protrusion are the most important symptoms [9]. Up to 50% of women have been reported to develop PFD [10]. The widening of the pelvic floor hiatus and descending of the pelvic floor below the pubococcygeal line with specific organ prolapsing has been considered the main cause of PFD which usually associated with obesity, pregnancy, childbirth, or inherited deficiency of collagen which induces weakness of the connective tissue [9]. Benzodiazepines were considered the best treatment for the acute management of the severe pain associated with PFD, particularly diazepam (DZ). DZ is a long-acting benzodiazepine which recently was used for the management of PFD due to its sedative muscle relaxant and amnestic properties. Currently, vaginal DZ suppositories or tablets are prescribed to relieve the pain of interstitial cystitis, vulvar pain, sexual pain, and to decrease the sedation of parenteral or oral DZ [11]. Copra et al. developed vaginal pessaries loaded with DZ for the treatment of interstitial cystitis [12]. Balkis reported that the best release of DZ was achieved by the use of either glycerol gelatin or glycerol-PEG1540 suppository base [13]. The fundamental goal of this research investigation was the development of DZ-loaded vaginal beads as a novel treatment approach for PFD and the decrease of the sedation associated with oral or intravenous DZ.

MATERIALS AND METHODS

Materials

DZ, SA, PL, PC, and XG were kindly supplied by EIPICO Company, Egypt. Calcium chloride (CaCl_2), acetonitrile, methanol, and potassium dihydrogen orthophosphate were purchased from El-Nasr Company, Egypt. All other chemicals were of analytical grade.

Methods

Formulation of alginate beads

Ionotropic gelation method was used for the preparation of DZ-load ed beads [14,15]. Accurately weighed amount of SA was dissolved in phosphate buffer pH 6.5 by stirring under gentle heating. PL or other mucoadhesive polymers was added to this solution. After 5 min, DZ was added with constant stirring for homogenous distribution. The formed dispersion was sonicated for 20 min to remove any air bubbles. Then, the solution was set aside for another 20 min. The resulted
solution was dropped through a 22-gauge syringe with 0.8-mm internal diameter from a distance of 5 cm into 100 ml CaCl\(_2\) solution at different concentrations as a cross-linking agent at 400 RPM and ambient room temperature. The drop-in rate was kept constant (2 ml/min). The formed beads were left for 30 min under stirring as a curing time. Finally, the beads were filtered, washed, and dried in a vacuum oven at 40°C for 24 h. The composition of the successful DZ beads is given in Table 1.

Characterization of DZ loaded beads

**Particle size analysis**

The particle size of different beads formulations was determined by counting 100 beads using a calibrated optical microscope [16].

**pH**

20 mg of each formula was allowed to swell into 20 ml distilled water for 5 h. Then, the pH of the solution was determined using a pH meter (JENCO Model-5005 USP).

**Yield percentage**

The prepared alginate beads were filtered, collected, dried, and weighed. The total yield percentage was determined based on the dried weight of the drug and the polymers according to the following equation.

\[
\text{Yield percentage} = \frac{\text{Actual weight of the product}}{\text{Total weight of the drug and excipient}} \times 100
\]

**Entrapment efficiency**

Accurately weighed quantity of the formed beads equivalent to 10 mg of the drug was crushed, suspended in 100 ml phosphate buffer pH 6.5 and stirred at 100 RPM at 25°C in a thermostatic shaker water bath ([JLAB SW-20 C, Germany]) till equilibrium [15]. The solution was filtered, suitably diluted and the drug entrapped was determined by RP-C18 HPLC method using a mobile phase consisting of acetonitrile, methanol, and 1% phosphate buffer (pH 3) in a ratio of 18:58:24 (v/v/v) at a flow rate of 1 ml/min and the effluent was monitored using UV detection at 232 nm [17] against drug free beads as a blank.

\[
\text{Entrapment efficiency} = \frac{\text{Actual drug content}}{\text{Theoretical drug content}} \times 100
\]

**In vitro bioadhesion**

In vitro bioadhesion of the formulated beads was determined by a previously reported method [8]. The vagina of the overnight fasted female rabbits was removed, cut into square pieces measuring 2 cm×2 cm, and rinsed with phosphate buffer pH 6.5. 50 mg of the prepared alginate beads were placed onto the vaginal mucosa, at ambient room temperature for 30 min. The vaginal mucosa was then rinsed with phosphate buffer pH 6.5 and the washings were dried in a vacuum oven at 40°C. The percentage of the bioadhesion was determined according to the following equation:

\[
\text{Bioadhesion percent} = \frac{\text{The adhered beads}}{\text{The applied beads}} \times 100
\]

**Swelling percentage**

About 50 mg of each formula was accurately weighed and placed in a wire basket of USP dissolution apparatus type II. The basket was then immersed in 500 ml phosphate buffer pH 6.5, at 37°C and 50 RPM. The beads were removed after 1 h, dried with tissue paper, and weighed [18,19]. Swelling percentage was determined according to the following equation:

\[
\text{Swelling (X)} = \frac{(W_f - W_0)}{W_0} \times 100
\]

Where, \(W_o\) is the weight of the dried beads and \(W_f\) is the weight of the swollen beads after 1 h.

**In vitro disintegration of the capsules**

To facilitate the administration of the prepared beads, they were filled into hard gelatin capsules. Watch glass method was used for the determination of the in vitro disintegration of the capsules. One capsule was placed at the center of a watch glass (11 cm in diameter). The watch glass was placed in a thermostatic shaker water bath at 37°C and 50 RPM. About 4 ml phosphate buffer pH 6.5 was added to the capsule. The time at which the beads were released from the capsule was the disintegration time [8].

**In vitro drug release study**

In vitro drug release studies were carried out in 900 ml phosphate buffer pH 6.5 at 37°C and 100 RPM using USP dissolution apparatus type II (basket type). Accurately weighed quantity of each formula equivalent to 10 mg DZ was placed into the basket. 3 ml samples were removed hourly for 8 h and replaced with an equal volume of a fresh buffer maintained at the same conditions. The withdrawn samples were filtered, suitably diluted and the drug content was determined by RP-C18 HPLC method at 232 nm [20].

**Ex vivo drug permeation study**

In this study, Franz diffusion cell was used. The model mucosal membrane was the rabbit vaginal mucosa. The vaginal mucosa was washed with phosphate buffer pH 6.5 and mounted between the receptor and the donor compartments. The receptor compartment was filled with 25 ml phosphate buffer pH 6.5, kept at 37°C, and stirred at 50 RPM with 3 ml samples were removed hourly for 8 h and replaced with fresh samples maintained at the same conditions. The permeated quantity of the drug was determined at 232 using RP-C18 HPLC method.

**Stability study**

The optimized DZ-loaded beads formulations were subjected to the stability study according to the ICH guidelines. The optimized formulations were placed at 40°C and 75±1 RH for 3 months. The samples were removed monthly and evaluated for their physical appearance, entrapment efficiency, and cumulative release percentage after 8 h [21].

**Pharmacological study**

To estimate the sedative action of the prepared intravaginal DZ-loaded alginate beads, the optimized formulations F3, F7, F9, and F11 were subjected to some behavioral psychopharmacological tests.

**Study design**

White albino female mice (20–25 g) were used in this study. Animals were housed at the standardized condition of the animal house of Faculty of Pharmacy, Zagazig University, Zagazig, Egypt. All animals were acclimatized and kept constant at ambient room temperature. All animal procedures were performed in accordance with the approved protocol for the use of the experimental animals set by the standing committee of the animal care of the faculty of pharmacy, Zagazig.

**Table 1: Composition of different formulations of DZ-loaded alginate beads**

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<td>1:1:2</td>
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<td>-</td>
<td>1</td>
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<td>1:1:1</td>
<td>-</td>
<td>-</td>
<td>1</td>
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<td>-</td>
<td>1</td>
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<td>1:1:2</td>
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</table>

DZ: Diazepam, SA: Sodium alginate, PL: Poloxamer 407, PC: Pectin, XG: Xanthan gum, CaCl\(_2\): Calcium chloride (cross-linking agent)
University, Egypt P12-12-2017. The animals were divided into seven groups, each of six mice. Group 1 act as a control and received distilled water (10 ml/kg, PO), Group 2 act as positive control and received DZ (3 mg/kg, IP), Group 3 act as positive control and received DZ (3 mg/kg, PO) [22], and Group 4, 5, 6, and 7 received the optimized formulations F3, F7, F9, and F11, respectively (3 mg/kg, intravenously).

**Traction test**
Mice were suspended individually from their anterior limbs to a wire that was horizontally stretched. The mice that were under sedation were the mice that failed to reestablish, at least one of their posterior limbs reached the wire. The reaction was considered positive when the mice performed immediate reestablishment. Otherwise, the reaction was considered negative [23].

**Hole board test**
The mice were placed individually in the center of perfomed wood board measuring 40 cm x 40 cm x 25 cm, in which evenly spaced holes were made. The number of the head dips was counted during a period of 5 min. The number of head dips was determined by the number of explored holes [24].

**Kinetic analysis**
In vitro release and ex vivo permeation data were subjected to theoretical analysis to estimate the kinetic order according to Higuchi diffusion, zero order, first order, Korsmeyer–Peppas, and Hixson and Crowell [25].

**Statistical analysis**
All experiments were run in triplicates. The obtained results were expressed as mean ± standard deviation. ANOVA was used to determine the significance. The results were considered statistically significant at p<0.05.

**RESULTS AND DISCUSSION**

DZ-loaded alginate beads were prepared by an ionotropic gelation method using SA alone or in combination with other mucoadhesive polymers such as PL, PC, or XG.

**Particle size**
The mean particle size of the prepared beads is illustrated in Table 2. The mean bead size was from 415±2.7 µm to 502±2.7 µm. The polymer concentration had an obvious effect on the bead size. It was found that as DZ:SA ratio increased from 1:1 to 1:5, there was a significant increase in the mean bead size from 431±2.3 µm (F1) to 502±2.7 µm (F3). The increase in the bead size with the increase in the alginate concentration may be ascribed to the increase in the viscosity of the polymer dispersion [26]. These findings were in a good correlation with Kashid *et al.* who found that with an increase in DS: polymers ratio from 1:2.5 to 1:7.5, there was a significant increase in the yield from 85.27% to 90.8%, respectively [32].

It is clear from the results in Table 2, that the concentration of the cross-linking agent played a crucial role in the formulated beads size. It was found that as the concentration of CaCl₂ was increased from 1% to 2%, there was a significant reduction in the mean beads size from 431±2.3 µm (F1) to 415±2.7 µm (F4). Further increase of CaCl₂ concentration to 3%, resulted in an additional reduction of the beads size to 395±3.3 µm (F3). This was in a great accordance with Manjanna *et al.*, who reported a significant reduction of the bead size from 734.1±0.54 to 688.5±1.25 µm with an increase in CaCl₂ concentration from 2% to 5% [28]. This could be discussed on the basis that ionic gelation occurred immediately when a drop of alginate dispersion came into contact with calcium ions. As calcium ion penetrated the interior of the droplets, water was squeezed out of them resulting in the contraction of the formed beads. The high concentration of the cross-linking agent favors the shrinkage of the resulting beads and eventually a significant reduction in the mean bead size [29].

The addition of PL, PC, or XG to the alginate dispersion in a ratio of 1:1 (DZ:SA:additional polymer), resulted in a significant increase in the beads size to 455±2.8 µm (F6), 485±3.1 µm (F8), and 485±3.6 µm (F10), respectively, compared to F1 (431±2.3 µm). A further increase in the ratio to 1:1:2, resulted in a corresponding increase in the beads size to 470±4.2 µm (F7), 496±2.8 µm (F9), and 515±2.8 µm (F11), respectively. Sankula *et al.* reported an increase in the mean bead size with the addition of Chp974 to SA dispersion [30]. Pal *et al.* found that the increase in the viscosity of the polymer dispersion resulted in the formation of big size droplets which contained more mass and consumed more time to fall from the syringe than the lower viscous polymer dispersion [31].

**Yield percentage**
Results in Table 2 show the production yield percentage of the different beads formulations. It was found that there was a significant increase in the yield from 68.2±1.7% (F1) to 85.1±1.4% (F3), with the increase in DZ:SA ratio from 1:1 to 1:5. Jelvehgari *et al.* found that with an increase in DS:polymer ratio from 1:2.5 to 1:7.5, there was a significant increase in the yield from 85.27% to 90.8%, respectively [32].

An increase in the concentration of CaCl₂ from 1% to 2%, resulted in a significant increase in the yield from 68.2±1.7% (F1) to 78.2±1.8% (F4). A further increase in the concentration of cross-linking agent to 3% had a nonsignificant effect on the yield.

The addition of PL, PC, or XG to the alginate dispersion in a ratio of 1:1:1 (DZ:SA:additional polymer), resulted in a significant increase in the yield to 85.4±1.8% (F10), comparatively, to F1 (68.2%±1.7) [26]. A further increase in the yield from 85.4±1.8% to 87.5±2.1% (F11), respectively. These results were in a good agreement with was found by Sankula *et al.* [30].

**Entrapment efficiency**
The entrapment efficiencies of the prepared beads are given in Table 2. There was a significant increase in the entrapment efficiency from 68.2%±1.7% to 85.4±1.8% (F4). Further increase in the concentration of cross-linking agent to 3% had a nonsignificant effect on the yield.

The addition of PL, PC, or XG to the alginate dispersion in a ratio of 1:1:1, resulted in a significant increase in the yield to 77.2±2.1% (F6), 75.6±1.5% (F8), and 80.4±1.8% (F10), respectively, compared to F1 (68.2%±1.7). A further increase in the ratio to 1:1:2, resulted in a clear significant increase in the yield to 85.4±1.8% (F7), 87.1±2.1% (F9), and 87.5±2.1% (F11), respectively. These results were in a good agreement with was found by Sankula *et al.* [30].

**Table 2: Particle size, yield percentage, and entrapment efficiency of different DZ-loaded alginate beads**

<table>
<thead>
<tr>
<th>Formula code</th>
<th>Bead size (µm)±SD*</th>
<th>Yield percentage ± SD*</th>
<th>Entrapment efficiency (%)±SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>431±2.3</td>
<td>682±1.7</td>
<td>65.6±1.6</td>
</tr>
<tr>
<td>F2</td>
<td>470±3.1</td>
<td>756±2.1</td>
<td>71.2±2.1</td>
</tr>
<tr>
<td>F3</td>
<td>502±2.7</td>
<td>851±1.4</td>
<td>78.9±3.1</td>
</tr>
<tr>
<td>F4</td>
<td>415±2.7</td>
<td>782±1.8</td>
<td>70.2±2.1</td>
</tr>
<tr>
<td>F5</td>
<td>395±3.3</td>
<td>772±2.1</td>
<td>78.2±1.8</td>
</tr>
<tr>
<td>F6</td>
<td>455±2.8</td>
<td>772±2.1</td>
<td>68.2±2.1</td>
</tr>
<tr>
<td>F7</td>
<td>470±4.2</td>
<td>854±1.8</td>
<td>70.1±1.6</td>
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<td>F8</td>
<td>458±3.1</td>
<td>756±1.5</td>
<td>70.6±2.4</td>
</tr>
<tr>
<td>F9</td>
<td>496±2.9</td>
<td>871±2.1</td>
<td>75.4±1.8</td>
</tr>
<tr>
<td>F10</td>
<td>485±3.6</td>
<td>804±1.8</td>
<td>72.6±2.6</td>
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<td>F11</td>
<td>515±2.8</td>
<td>875±2.1</td>
<td>78.4±1.3</td>
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</table>

*Each result is the mean of three determinations ± standard deviation. DZ: Diazepam.
1:1 to 1:5. An increase in SA concentration was accompanied with a progressive increase in the beads size which entrapped more drugs. The increase in alginate concentration resulted in the formation of a dense matrix structure which reduced the loss of the drug during the curing time [28].

An increase in CaCl₂ concentration from 1% to 2%, resulted in a significant increase in the entrapment of the drug from 65.6±1.6 (F1) to 70.2±2.1 (F4). Further increase to 3%, resulted in a further increase in the drug entrapment to 78.2±1.8 (F5). As the concentration of the cross-linking agent increased, the cross-linking of the polymer, and the compactness of the formed insoluble dense polymer matrix increased. This resulted in the entrapment of more drug and the reduction of the drug diffusion during the curing time [28].

The addition of PL at a ratio of 1:1:1 had a nonsignificant effect on the entrapment efficiency, but further increase in the ratio to 1:1:2 resulted in a significant increase to 70.1±1.6 (F7).

The addition of either PC or XG to the alginate dispersion at a ratio of 1:1:1, resulted in a significant increase in DZ entrapment to 70.6±2.4 (F8) and 72.6±2.6 (F10), respectively, compared to F1 (65.6±1.6). A further increase in the entrapment to 75.4±1.8 (F9) and 78.4±1.3 (F11) was observed with the increase of the ratio to 1:1:2, respectively. The significant increase in DZ entrapment within the beads with the addition of the associated polymers could be attributed to the increase in the viscosity of the polymer dispersion, which resulted in a larger dense matrix beads that entrapped more drug and prevent its diffusion during the curing time [26,28].

**pH**

The pH of all prepared beads formulations was in the range from 6.1±0.2 to 6.8±0.6 which ensured that the prepared beads were non-irritating and safe to be administered into the vagina (Table 3).

**Swelling index**

The results in Table 3 depict the swelling percentages of different beads formulations after 1 h. An increase in DZ:SA ratio from 1:1 to 1:5, resulted in a significant increase in the swelling percentage from 53.4±3.1(F1) to 68.4±3.5 (F3), respectively. This was in a good agreement with Iswariya et al. [33].

Swetha et al. found that when the concentration of Cbp934 increased, the swelling percentage of celecoxib-loaded beads significantly increased [34].

There was a significant reduction in the swelling percentage from 53.4±3.1 (F1) to 31.2±1.7 (F4), with an increase in the concentration of CaCl₂ from 1% to 2%, respectively. Further increase in CaCl₂ concentration to 3%, resulted in an additional reduction to 23.2±2.1 (F5). Jahan et al. reported a significant reduction in the swelling percentage of theophylline-loaded SA beads from 12.3% to 5.9%, with an increase in CaCl₂ concentration from 5% to 10%, respectively, [35]. An increase in the concentration of the cross-linking agent increased the extent of the polymer cross-linking which decreased the polymer chain length. The decrease in the polymer chain length decreased the ability of the polymer to expand and swell [36].

The addition of PL, PC, or XG to the alginate dispersion in a ratio of 1:1:1, resulted in a significant increase in the swelling percentage to 60.2±1.8 (F6), 73.1±2.5 (F8), and 72.8±3.1 (F10), respectively, compared to F1 (53.4±3.1). Further increase in the swelling percentage to 66.3±1.5 (F7), 85.2±3.7 (F9), and 83.2±2.8 (F11), respectively, was observed with an increase in the ratio to 1:1:2. This could be ascribed to the increase in the hydrophilicity of the polymer matrix [37].

**Bioadhesion strength**

Table 3 shows the bioadhesion strength of the different beads formulations. The results demonstrated an increase in the percentage of beads that adhered to the rabbit vaginal mucosa from 71.5±1.3 (F1) to 82.1±1.8 (F3) with an increase in DZ:SA ratio from 1:1 to 1:5, respectively. There was a significant increase in the maraviroc alginate microspheres mucoadhesion percentage after 4 h from 55.3±3.1 to 78.2±0.8 with an increase in the drug to polymer ratio from 1:1 to 1:2, respectively [21]. An increase in the concentration of SA resulted in an increase in the amount of free carboxylic group which was responsible for the binding to the sialic acid group of the vaginal mucosa [27].

An increase in the cross-linking agent concentration from 1% to 2%, resulted in a significant decrease in the bioadhesion strength from 71.5±1.3 (F1) to 66.2±1.5 (F4). A further decrease in the bioadhesion strength to 61.5±2.4 (F5) was obtained with a further increase in the cross-linking agent concentration to 3%. The reduction of the bioadhesion strength could be discussed on the basis of the surface charge density. An increase in the extent of cross-linking resulted in a significant decrease in the surface-negative charge on the prepared alginate beads and consequently decreased the bioadhesion strength [38]. On incorporation of PL, PC, or XG within the alginate beads in a ratio of 1:1:1, the bioadhesion strength of the resultant beads increased to 74.2±1.8 (F6), 80.2±2.1 (F8), and 79.5±2.1 (F10), respectively, compared to F1 (71.5±1.3). A further increase in the polymer ratio to 1:1:2, resulted in a further increase to 78.1±1.5 (F7), 87.6±3.1 (F9), and 86.3±2.1 (F11), respectively. This could be attributed to the increase of either hyaluronic group content or carboxylic group content or both of the prepared beads which resulted in more hydrogen bonding with the substrate [33].

**In vitro disintegration of the capsules**

It was found that the prepared beads formulations were completely released from the capsules after approximately 10 min.

**In vitro release study**

The cumulative release profiles of the DZ from the prepared beads were depicted in Figs. 1-5. It was clear from Fig. 1 that the concentration of SA had a significant influence on the DZ release percentage after 8 h. The cumulative percentage of DZ significantly decreased from 92.4% (F1) to 75.6% (F3), with an increase in DZ:SA ratio from 1:1 to 1:5, respectively (Fig. 1). This was in a good correlation with Rasel and Hasan, who found that the release of didofenac sodium significantly decreased from 76.7% to 69.1%, as the amount of SA increased from 2 g to 4 g [15]. This could be attributed to the formation of a tight junction between the glucuronic acid residues as a result of cross-linking. An increase in SA concentration led to an increase in the number of cross-linking points, so more drug would be entrapped and eventually a significant reduction in the total drug release from the resulting beads [39]. This could also be attributed to the formation of gelatinous mass on the surface of the formed beads on contact with the dissolution medium which hindered further penetration of the dissolution medium and increased the diffusional pass length [3,40].

As the concentration of CaCl₂ increased from 1% to 2%, there was a significant reduction in the DZ cumulative release percentage from...
92.4% (F1) to 85.1% (F4); further increase in the concentration of cross-linking agent to 3% induced further reduction in the percentage released to 76.5% (F5) (Fig. 2). An increase in the cross-linking agent concentration, led to the formation of more rigid gel structure with smaller pore size, which retarded the penetration of the dissolution medium into the beads which in turn decreased the total amount of the drug released [15]. This was in a good correlation with Mandal et al. who reported a significant decrease in the amount of trimetazidine dihydrochloride release from 38% to 30% with an increase in CaCl2 concentration from 1% to 3% [41].
The incorporation of PL at 1:1:1, significantly decreased the DZ release to 86.1% (F6) compared to F1 (92.4%). A further increase in the ratio to 1:1:2, resulted in a further reduction in the release to 80.2% (F7) (Fig. 3). Parhi and Suresh found that an increase in PL concentration from 0% to 1%, resulted in a significant decrease in metoprolol succinate from 93.13% to 76.5%, respectively [20].
addition of PC in a ratio of 1:1:1 significantly reduced the cumulative percentage after 8 h from 92.4% (F1) to 85.1% (F8). Further reduction in the release to 79.2% (F9) was obtained with further increase in the PC ratio to 1:1:2 (Fig. 4). Khan et al. found that verapamil HCL release from PC-alginate beads was lesser than its release from alginate beads only [14].

XG significantly decreased the DZ release percentage from 92.4% (F1) to 81.9% (F10) at 1:1:1. Further increase in the ratio to 1:1:2, resulted in a further reduction in the drug release to 72.6% (F11) (Fig. 5). Kulkarni et al. [42] reported a sustained release of glipizide from XG-chitosan microbeads.

These results could be ascribed to the formation of less porous gel mass by either PL, PC, or XG along with the longer diffusion path length formed by the higher concentration of the incorporated polymers.

**Ex vivo drug permeation study**

Fig. 6 demonstrates the permeation profile of DZ through the rabbit vaginal mucosa. F3, F7, F9, and F11 were selected for the study based on their in vitro release profiles, entrapment efficiencies along with their bioadhesion strengths. It was found that about 74.8%, 71.1%, 68.6%, and 63.4% of the loaded dose permeated through the rabbit vaginal mucosa from F7, F9, F3, and F11, respectively. Based on the release and permeation data, it was found that there was a good correlation between the in vitro release and ex vivo permeation of DZ which emphasized the effective therapeutic action of the prepared beads.

**Kinetic analysis**

The in vitro drug release and ex vivo drug permeation of the tested formulations followed Higuchi diffusion model.

**Stability study**

Based on the release profile, entrapment efficiency, and bioadhesion strength; F3, F7, F9, and F11 were selected for conducting the stability study by storing the selected formulations at 40°C and 75±1 RH for 3 months. There was a negligible change in the tested parameters which ensured the good stability of the prepared beads (Table 4).

**Traction test**

Table 5 illustrates the reestablishment times performed by the studied animal groups. It was obvious that Group 1 reestablished immediately after 2 s. Group 2 and 3 responded after 30 and 22 s, respectively. Group 4, 5, 6, and 7 performed reestablishment after 11, 10, 13, and 11 s respectively. The significant reduction in the reestablishment time in the groups that received intravaginal alginate beads compared to the animal groups which received either IP or PO DZ indicated that the prepared alginate beads reduced the sedative action of the parenteral or oral DZ.

**Hole board test**

The results in Table 5 show the number of the explored holes of the studied groups. The significant increase in the number of head dips to 11, 10, 9, and 12 for F3, F7, F9, and F11 respectively, compared to either IP DZ (1) or PO DZ (4), suggested also that, the formulation of DZ into intravaginal alginate beads successfully decreased its sedative action.

**CONCLUSION**

DZ has been successfully formulated into intravaginal beads using a variety of bioadhesive polymers which were easily administered into the vagina with minimum central nervous system side effects. The obtained results are promising to develop a clinical study to establish its therapeutic efficacy and safety on the PFD patients.

**AUTHOR CONTRIBUTION**

All the work has been conducted by the corresponding author.

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**Table 4: Stability study of the optimized beads at 40°C and 75±1 RH**

<table>
<thead>
<tr>
<th>Formula code</th>
<th>Time (months)</th>
<th>Entrapment efficiency (%)±SD*</th>
<th>Bioadhesion strength (%)±SD*</th>
<th>Cumulative release (%)±SD*</th>
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<tbody>
<tr>
<td>F3</td>
<td>0</td>
<td>78.2±3.1</td>
<td>82.1±1.8</td>
<td>75.6±3.4</td>
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<tr>
<td></td>
<td>1</td>
<td>77.2±2.7</td>
<td>80.2±1.5</td>
<td>77.2±2.7</td>
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<tr>
<td></td>
<td>2</td>
<td>79.8±1.8</td>
<td>83.4±2.1</td>
<td>76.8±4.1</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>76.9±3.2</td>
<td>82.9±2.7</td>
<td>74.1±3.1</td>
</tr>
<tr>
<td>F7</td>
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<td>80.9±3.1</td>
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<tr>
<td></td>
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<td>71.2±2.9</td>
<td>79.5±2.1</td>
<td>81.2±3.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>71.6±2.5</td>
<td>80.1±1.9</td>
<td>78.4±2.7</td>
</tr>
<tr>
<td>F9</td>
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<td>75.4±1.8</td>
<td>87.6±3.1</td>
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<tr>
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<td>86.3±2.1</td>
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<td>77.8±2.1</td>
<td>88.1±2.7</td>
<td>71.6±3.6</td>
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</table>

*Each result is the mean of three determinations±standard deviation.

---

**Table 5: Sedative action of the optimized diazepam-loaded alginate beads**

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Formula received</th>
<th>Traction test (reestablishment time (S)±SD*)</th>
<th>Hole board test (explored holes during 5 min)±SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Distilled water</td>
<td>2 ± 0.02</td>
<td>18 ± 1.2</td>
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<tr>
<td>2</td>
<td>Diazepam (IP)</td>
<td>30 ± 0.4</td>
<td>1 ± 0.8</td>
</tr>
<tr>
<td>3</td>
<td>Diazepam (PO)</td>
<td>22 ± 0.6</td>
<td>4 ± 0.1</td>
</tr>
<tr>
<td>4</td>
<td>F3</td>
<td>11 ± 0.09</td>
<td>11 ± 0.2</td>
</tr>
<tr>
<td>5</td>
<td>F7</td>
<td>10 ± 0.3</td>
<td>10 ± 0.8</td>
</tr>
<tr>
<td>6</td>
<td>F9</td>
<td>13 ± 0.5</td>
<td>9 ± 1.1</td>
</tr>
<tr>
<td>7</td>
<td>F11</td>
<td>11 ± 0.6</td>
<td>12 ± 0.9</td>
</tr>
</tbody>
</table>

*Each result is the mean of six determinations±standard deviation.
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CONFLICT OF INTEREST

The author declares no conflict of interest.

REFERENCES