Promising nanoparticulate system for topical delivery of diphenhydramine hydrochloride: In-vitro and in-vivo evaluation

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1. Introduction

The first-generation of antihistamines, also known as sedating antihistamines, are highly potent competitive inhibitors for histamine, acting on the histamine H1-receptor sites distributed throughout the body [1]. They are used in treatment of the allergic rhinitis, allergic conjunctivitis also in vertigo, motion sickness and local skin reactions [2].

When orally taken, they are easily able to penetrate the blood brain barrier and occupy H1-receptor sites in the brain, resulting in fatigue, drowsiness, and performance impairment. Meanwhile conventional topical gels of diphenhydramine hydrochloride showed less patient compliance due to increased frequency of application on the skin.

Diphenhydramine hydrochloride, histamine H1– receptor antagonist, is widely used as antiallergic and antiemetic drug in many pharmaceutical preparations, as ingredient in common cold preparations. It passes metabolism, sustained and controlled delivery over a prolonged period of time, improved patient acceptance and compliance, direct access to target or diseased site, ease of dose termination in the event of disturbance, CNS or anticholinergic adverse effects as dry mouth resulting in a less patient compliance with the therapy. The aim of the current study was to prepare diphenhydramine hydrochloride loaded nanoparticulate system, using chitosan as a natural polymer for topical application. Eight formulae were prepared adopting 2^3 factorial design. Diphenhydramine hydrochloride loaded nanoparticles were prepared by ionic gelation technique using chitosan and sodium tripolyphosphate (TPP). The formulae were evaluated regarding TEM, entrapment efficiency, particle size, zeta potential, in-vitro release, DSC, XRD, kinetics study, and in-vivo study regarding skin irritation test and histopathological examination using rats. The results revealed that the entrapment efficiency was significantly increased when increasing the chitosan concentration, the drug to polymer ratio and the chitosan to TPP (w/w) ratio. The particle size was significantly increased when increasing the chitosan concentration, the drug to polymer ratio but significantly decreased when increasing the chitosan to TPP (w/w) ratio. The zeta potential was significantly increased by increasing the chitosan concentration, the drug to polymer ratio and the chitosan to TPP (w/w) ratio. The in-vitro release study showed prolongation of drug release up to 6hrs. A comparison was made between the candidate formula (F8) (0.375% of diphenhydramine hydrochloride, drug to polymer 1:2, chitosan concentration 0.75% and chitosan to TPP (w/w) 5:1) and the marketed gel. The skin irritation test of F8 revealed its dermal safety and the statistical analysis revealed significant increase in its antihistaminic activity with reduction in the wheal area (from 150 mm^2 ± 7.8 to 43.6 mm^2 ± 4.9) when compared to the marketed gel (from 155 mm^2 ± 6.1 to 82.1 mm^2 ± 8.54). A value of r = 0.97704 suggested a good correlation between the in vitro-in vivo data of the candidate formula. The results revealed that the developed nanoparticles could have a potential for topical delivery of diphenhydramine hydrochloride.
any adverse reactions either systemic or local and provide an alternative when oral dosing is not possible (in unconscious or nauseated patients) [3]. For these benefits, topical application of first generation antihistamines is most favorable. Sanna et al. previously prepared diphenhydramine hydrochloride in different topical vehicles as microemulsion, microemulsion with silica and carbopol cream [4] and Zaki Rizkalla et al. prepared hydroxyzine hydrochloride loaded in microsponges [5].

Biodegradable polymeric nanoparticles have attracted prominent interest in the past few decades as a novel drug carrier of various therapeutic agents for controlled drug delivery through the topical route, especially to the viable epidermis layer where inflammatory reactions take place [6]. Chitosan due to its unique physico-chemical and biological properties is an attractive material for use in various applications. Chitosan is an effective biodegradable polymer due to its bio-compatibility, biodegradability and low-toxicity. It also has antimicrobial activity and low immunogenicity. It also shows wound healing capacity and hemostatic activities [7].

To the best of our knowledge no previous attempts were done to prepare diphenhydramine hydrochloride loaded chitosan nanoparticles for topical delivery, by ionic gelation technique, to overcome the CNS adverse reactions of the oral therapy, to improve the patient compliance when topically applied with less frequency of application and to provide targeted therapy with enhanced skin delivery and prolonged antiallergic effect.

2. Materials and method

2.1. Materials

Diphenhydramine Hydrochloride was received as a gift from Pharma Sweden, Egypt. Sodium tripolyphosphate granular was purchased from Alfa Aesar GmbH & COKG, Germany. Chitosan 85%DA was purchased from Meron, India. Histamine dihydrochloride was purchased from Sigma-Aldrich Co., St. Louis, USA. All other reagents and chemicals used were of analytical reagent grade. Insect bite (diphenhydramine hydrochloride 1%, topical gel, Pharmaia pharmaceuticals, Egypt, marked gel).

2.2. Methodology

2.2.1. Preparation of diphenhydramine hydrochloride loaded chitosan nanoparticles

Diphenhydramine hydrochloride loaded chitosan nanoparticles were prepared by ionic gelation technique, using chitosan as coating material and sodium tripolyphosphate (TPP) as cross linking agent according to the method adopted by Leelapornpisid with slight modifications [8]. Chitosan was dissolved in aqueous medium at different concentrations, 0.5% and 0.75% w/w (5 mg/ml and 7.5 mg/ml respectively), with 1%v/v acetic acid. The drug at different ratios, drug to chitosan ratios: 1:1 and 1:2 (for 0.5% w/w chitosan concentration with drug: polymer ratio 1:1, 500 mg of drug was added in 100 ml of chitosan solution and for drug: polymer ratio 1:2, 250 mg of drug was added to 100 ml of chitosan solution. For 0.75% w/w chitosan concentration with drug: polymer ratio 1:1, 750 mg of drug was added to 100 ml chitosan solution and for drug: polymer ratio 1:2, 375 mg of drug was added to 100 ml chitosan solution), was added to the chitosan solution of different concentrations and was stirred using a magnetic stirrer (BOECO, Germany) until it completely dissolved. Sodium tripolyphosphate was separately dissolved in distilled water at concentration with drug: polymer ratio 3:1 and 5:1, as shown in Table [2]. Nanoparticle suspensions were stirred at 700 rpm using a magnetic stirrer (BOECO, Germany) for 60 min at ambient temperature before characterization.

2.2.2. Experiment design

A factorial design (2³) was adopted using the Design Expert® software (Version 7, Stat-Ease Inc., Minneapolis, MN) to study the effect of three factors as in Table [1]. These independent factors namely chitosan concentration, drug to polymer ratio and chitosan to TPP weight ratios. The responses were the % entrapment efficiency, mean particle size, zeta potential and 90% of drug release (the time taken to release 90% of the drug). Eight formulae were prepared as shown in Table [2].

3. Characterization and evaluation of diphenhydramine hydrochloride loaded chitosan nanoparticles

3.1. Transmission electron microscopy (TEM)

The morphological examination of nanoparticles was observed by transmission electron microscopy (Tecnai G20, Super twin, double tilt, FEI, Netherlands). The prepared nanoparticles were diluted with double distilled water and dropped on a copper grid with staining for 30 s and air dried at room temperature. Then, a drop of 0.2% phosphotungstic acid was added and stained for 30 s to be air-dried. Finally, the air-dried samples were directly placed under the TEM for observation [9].

Table 2

Composition of the prepared diphenhydramine hydrochloride loaded chitosan nanoparticles according to 2³ design.

<table>
<thead>
<tr>
<th>Formulation Code</th>
<th>Chitosan Concentration</th>
<th>Drug:Polymer (D:P)</th>
<th>Chitosan:TPP (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>0.5%a</td>
<td>1:1f</td>
<td>3:1c</td>
</tr>
<tr>
<td>F2</td>
<td>0.5%a</td>
<td>1:2e</td>
<td>3:1c</td>
</tr>
<tr>
<td>F3</td>
<td>0.5%a</td>
<td>1:1f</td>
<td>5:1b</td>
</tr>
<tr>
<td>F4</td>
<td>0.5%a</td>
<td>1:2e</td>
<td>5:1b</td>
</tr>
<tr>
<td>F5</td>
<td>0.75%a</td>
<td>1:1f</td>
<td>3:1c</td>
</tr>
<tr>
<td>F6</td>
<td>0.75%a</td>
<td>1:2e</td>
<td>3:1c</td>
</tr>
<tr>
<td>F7</td>
<td>0.75%a</td>
<td>1:1f</td>
<td>5:1b</td>
</tr>
<tr>
<td>F8</td>
<td>0.75%a</td>
<td>1:2e</td>
<td>5:1b</td>
</tr>
</tbody>
</table>

a The chitosan concentration was 5 mg/ml.

b The chitosan concentration was 7.5 mg/ml.
c The theoretical amount of drug was 500 mg/100 ml of chitosan solution.
d The theoretical amount of drug was 250 mg/100 ml chitosan solution.
e The theoretical amount of drug was 750 mg/100 ml of chitosan solution.
f The theoretical amount of drug was 375 mg/100 ml of chitosan solution.
g The volume of TPP solution was 85.33 ml, added to 100 ml of chitosan solution.
h The volume of TPP solution was 33.33 ml, added to 100 ml of chitosan solution.
i The volume of TPP solution was 83.33 ml, added to 100 ml of chitosan solution.
j The volume of TPP solution was 50 ml, added to 100 ml of chitosan solution.
3.2. Particle size, polydispersity index and zeta potential determination

Average particle size, polydispersity index (PDI) and zeta potential were determined using the Laser diffraction particle size analyzer, Master sizer (Malvern instrument Co., UK), at ambient temperature. Samples were properly diluted with deionized water before measurements. All measurements were performed in triplicate and results were reported as mean ± standard deviation [10].

3.3. Determination of entrapment efficiency

The diphenhydramine hydrochloride loaded chitosan nanoparticles were separated from the aqueous suspension by centrifugation (Hermle, Germany) at 10,000 rpm, 4 °C for 15 min. The amount of free diphenhydramine hydrochloride was measured in the clear supernatant by UV spectrophotometry (Shimadzu UV-1650 PC, Japan) at \( \lambda_{\text{max}} \) 258 nm [11]. The determination of entrapment efficiency was evaluated using the difference between initial amount of diphenhydramine hydrochloride and unentrapped amount in the supernatant [12,13]. The entrapment efficiency of diphenhydramine hydrochloride was calculated according to the following formula:

\[
\text{Entrapment efficiency} = \left( \frac{W_{\text{t}} - W_{\text{f}}}{W_{\text{t}}} \right) \times 100
\]

Where, \( W_{\text{t}} \) is the total initial amount of diphenhydramine hydrochloride and \( W_{\text{f}} \) is the amount of free diphenhydramine hydrochloride in the supernatant after centrifugation (Hermle, Germany). All measurements were performed in triplicate and results were reported as mean ± standard deviation.

3.4. In vitro release studies

The diphenhydramine hydrochloride loaded chitosan nanoparticles were separated from the aqueous suspension medium through centrifugation (Hermle, Germany). The study was done according to a method adopted by Tous et al. with slight modifications, to reduce errors in dilution [14]. 5 dialysis membrane tubing bags (cutoff between 12,000 and 14,000 Daltons, Carolina, North Carolina) were previously soaked in phosphate buffer pH 5.5 for 2 h; then each bag contained 2 ml of each formula of chitosan nanoparticles, tied and placed in 5 graduated plastic tubes, each tube contained 25 ml of phosphate buffer pH 5.5. These tubes were placed on the shaker (Heidolph, Germany), set at 50 rpm [15,16]. At appropriate time intervals (0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 6 h), the amount of the drug released at each interval in the release medium was evaluated by UV spectrophotometer at \( \lambda_{\text{max}} \) 258 nm [11], by removing 3 mL of the release medium from each tube and replacing with fresh medium of phosphate buffer solution. All measurements were performed in triplicate and the results were reported as mean ± standard deviation.

3.5. Differential scanning calorimetry (DSC)

DSC experiment, was carried out to determine the possible interactions between the drug (diphenhydramine hydrochloride) and the polymer (chitosan) used. Three mgs of diphenhydramine hydrochloride powder, plain lyophilized chitosan nanoparticles and lyophilized diphenhydramine hydrochloride loaded chitosan nanoparticles F8 (the candidate formula) were placed in an aluminum pan and heated at a rate of 10 °C/min, in an atmosphere of nitrogen up to a temperature of 400 °C [17,18]. The differential scanning calorimetry (DSC) thermograms were recorded on a differential scanning calorimeter (DSC-50, Shimadzu, Kyoto, Japan).

3.6. X-ray diffractionmetry (XRD)

The X-ray diffraction pattern of the plain chitosan nanoparticles, diphenhydramine hydrochloride loaded chitosan nanoparticles of the candidate formula and diphenhydramine hydrochloride powder were determined. The analysis was done by X-ray diffractometer (Rigaku Ultima IV, Tokyo, Japan) using Cu Kα radiation, at voltage of 40 kV and a current intensity of 30 mA, and were scanned over a 2 θ range of 5–90 [19].

3.7. In vivo evaluation

3.7.1. Animals

Male albino Wistar rats with average weight (200 gm ± 10 gm) were used for the in vivo evaluation [20,21]. The animal study protocol was reviewed and approved by the Institutional Ethical Committee, Faculty of Pharmacy, Cairo University -Cairo-Egypt, PI (930).

3.7.2. Preliminary study

The rats were housed in pairs for a week under controlled environmental conditions (temperature 25 ± 2 °C and 12-h light–dark cycle) [20]. All the animals received standard laboratory diet and water. Two days before the study, depletory cream was applied for 15 min on the dorsal region of the rat and then thoroughly removed to ensure complete removal of the hair [5].0.1 ml sterile saline solution of histamine dihydrochloride containing (10, 50, 100 and 200 mg/ml) was intradermally injected by diabetic insulin syringe into the dorsal side of the rat. The typical wheal appeared after 5 min of histamine injection. wheal diameter was traced after 10 min [5] and its area was calculated according to the law: \( A = \pi r^2 \).

3.7.3. Animal group study

In this study, 35 rats were used in a parallel design and were divided into five groups (n = 7/group) as follows:

- Group 1: received nothing.
- Group 2: animals as control injected intradermally with histamine.
- Group 3: animals topicaly treated with diphenhydramine hydrochloride loaded chitosan nanoparticles (F8) for sensitivity test.
- Group 4: animals injected intradermally with histamine and treated with diphenhydramine hydrochloride loaded Chitosan nanoparticles (F8).
- Group 5: animals injected intradermally with histamine and treated with the marketed gel, Insect bite (Pharaonia pharmaceuticals, Egypt) containing 1% diphenhydramine hydrochloride.

3.7.4. Skin sensitivity studies and histopathological examination

The prepared diphenhydramine hydrochloride loaded chitosan nanoparticle was topically and uniformly applied on the dorsal region of the rats and kept in contact for 4hrs [21]. After the visual examination of the treated skin, the animals were sacrificed by cervical dislocation method and the exposed dorsal surface was cut. Then, each specimen was fixed in 10% formalin; embedded in paraffin and micromet. The sections were stained with hematoxylin and eosin. Finally, the specimens were observed under a high-power light microscope (BM208 M, China) and were evaluated for their integrity.

3.7.5. Evaluation of antihistaminic activity of diphenhydramine hydrochloride loaded chitosan nanoparticles and the marketed gel and histopathological examination

An amount of 2 ml (equivalent to 2 gm) of the marketed gel containing 20 mg of diphenhydramine hydrochloride and 2 ml (equivalent to 2 gm) of the prepared diphenhydramine hydrochloride loaded chitosan nanoparticles containing 7.6 mg diphenhydramine hydrochloride were applied on the dorsal side of the rat skin after the appearance of the wheal due to histamine injection and the cutaneous skin reactions were evaluated at different time intervals 0.25, 0.5, 1, 1.5, 2 h,
comparing the responses between different groups. Then the percentage of wheal suppression was calculated according to the equation:

\[ E = \frac{w_0 - w_t}{w_0} \times 100 \]

Where \( E \) is the efficacy of the applied formula, \( w_0 \) is the baseline wheal area, and \( w_t \) is the wheal area after time \( t \) of the formula application [5]. Then the animals were sacrificed and histopathologically examined as mentioned before.

3.7.6. *In-vitro/in-vivo correlation*

The relationship between cumulative percent of drug released from the candidate formula (F8) and the percent of wheal reduction when applying the candidate formula on the rats was established to predict its therapeutic efficiency, by plotting the cumulative percent of drug released against the percent of wheal reduction to determine the correlation.

4. Results and discussion

4.1. Characterization of diphenhydramine hydrochloride loaded chitosan nanoparticles

4.1.1. Morphological examination of diphenhydramine hydrochloride loaded chitosan nanoparticles using transmission electron microscope (TEM)

The TEM image Fig. [1] of the best achieved formula, F8 (0.375% of diphenhydramine hydrochloride, 0.75% chitosan concentration, drug to polymer ratio 1:2 and chitosan to TPP weight ratio 5:1) clearly revealed that the formed nanoparticles were regular spherical uniform shaped nanoparticles with consistent structure. The diameter of the nanoparticles observed in TEM was smaller than that obtained by particle size analysis which might be due to the swelling property of chitosan in the aqueous media during sample preparation to be measured by the particle size analyzer, in contrast during TEM sample preparation, nanoparticles were dehydrated which resulted in smaller particle size [9].

4.1.2. Entrapment efficiency

The range of the entrapment efficiency of the prepared formulae was found to vary from 15.10% ± 0.90%–85.15% ± 0.41%, as represented in Table [3]. Increasing the chitosan concentration from 0.5% to 0.75% significantly increased the entrapment efficiency (\( P < 0.0001 \)). This may be due to an increase in viscosity of the chitosan solution by increasing the chitosan concentration, which prevented the drug from leaving the nanoparticles. The results were in accordance with Sinha et al. [22] and Avadi et al. [23]. Concerning the drug to polymer ratio, it was found that when increasing the ratio from 1:1 to 1:2, the entrapment efficiency significantly (\( P < 0.0001 \)) increased up to a particular ratio so that sufficient quantity of polymer will be available to entrap drug in solution. This was in agreement with Tummala [24]. For chitosan to TPP (w/w) ratio, it was found that increasing the ratio from 3:1 to 5:1, the entrapment efficiency increased significantly (\( P < 0.0001 \)). It was previously stated that at very low or high chitosan to TPP ratios either a clear solution was seen (almost no particle formation) or larger nanoparticles with a low colloidal stability were obtained, respectively [25], consequently high drug entrapment efficiency would be obtained with certain chitosan to TPP (w/w) ratio to prepare stable nanoparticles, which was in this study 5:1 (as in F8). The effect of the three different factors on the entrapment efficiency of diphenhydramine hydrochloride in the formed nanoparticles was illustrated in Fig. [2].

4.1.3. Particle size determination and polydispersity index

The range of the particle size of the prepared nanoparticles was found to vary from 656.10 nm ± 5.1% to 1168.33 nm ± 28.4% and the polydispersity index was ranging from 0.16 ± 0.26 to 0.67 ± 0.28, as represented in Table (3). Concerning the chitosan concentration, increasing the chitosan concentration from 0.5% to 0.75% significantly increased the particle size (\( P < 0.0001 \)) which was in agreement with Leelapornpisid [8], Sarah et al. [26] and Perinelli et al. [27]. As for the drug to polymer ratio, it was previously reported that increasing drug concentration led to an increase in the particle size [28] and addition of a drug to be encapsulated increased the size of the formed nanoparticles compared to the size of the plain nanoparticles [29]. The particle size of the unloaded chitosan nanoparticles (plain F8) (0.75% chitosan concentration and chitosan to TPP weight ratio 5:1) was found to be 580.1 nm ± 9.2 and increased to 778.07 nm ± 11.22 upon addition of the diphenhydramine hydrochloride (around 25.4% increase in the size of loaded chitosan nanoparticlesF8). This particle size increase might be due to reduction in the ionic interaction between chitosan and TPP during the addition of the drug to prepare chitosan nanoparticles loaded with the drug [30]. Meanwhile increasing the drug to polymer ratio from 1:1 to 1:2, the size of the nanoparticles increased significantly (\( P < 0.0001 \)). Regarding the chitosan to TPP ratio (w/w), it was found that the particle size significantly (\( P < 0.0001 \)) decreased by increasing the ratio from 3:1 to 5:1 which was in accordance with Leelapornpisid [8] and Perinelli et al. [27] as 3:1 ratio, the amount of sodium tripolyphosphate was in excess leading to decreased zeta potential and consequently larger nanoparticles than that of ratio 5:1, were formed. Also Stoica and Ion [31] reported as chitosan-TPP mass ratio declined, the available quantity of sodium tripolyphosphate increased and that excess sodium tripolyphosphate would link the mono nanoparticles to form larger nanoparticles. The effect of the three factors on the particle size of the formed chitosan nanoparticles was illustrated in Fig. [3].

4.1.4. Zeta potential

Zeta potential represents the stability of the particulate system, as the value of zeta potential increases, the electrostatic repulsion interactions between the particles will be greater, and thus the stability of the particles will increase [32]. Particles with zeta potentials more positive than +20 mV or more negative than −20 mV are normally considered stable [33].

The range of zeta potential of the prepared formulae varied from +12.9 mV ± 5.13 to +25.2 mV ± 2.63. By increasing the chitosan concentration it was found that the zeta potential of the colloidal system increased significantly (\( P < 0.0001 \)) which was in agreement with Avadi et al. [23] who stated that the residual amine groups of chitosan would be responsible for the positive zeta potential values and same results were reported with Ekinci et al. [10] and Nagarajan et al. [34]. For the drug to polymer ratio, higher zeta potential values were obtained (\( p < 0.0001 \)) when increasing the ratio from 1:1 to 1:2 which may be due to presence of sufficient positive charges that increased the electrostatic repulsion between formed particles thus increased zeta potential [23]. Concerning the chitosan to TPP (w/w) ratio, as the ratio increased from 3:1 to 5:1 the zeta potential significantly increased.
Table 3
Average entrapment efficiency, particle size, polydispersity index (PDI), zeta potential and T90% of drug release of the prepared diphenhydramine hydrochloride loaded chitosan nanoparticles.

<table>
<thead>
<tr>
<th>Formulation code</th>
<th>Chitosan concentration (D:P)</th>
<th>Drug:polymer (D:P)</th>
<th>Chitosan:TPP (W/W)</th>
<th>Entrapment efficiency%</th>
<th>Particle size (nm)</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
<th>T90% of drug release (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>0.5%</td>
<td>1:1</td>
<td>3:1</td>
<td>15.10 ± 0.9</td>
<td>924.8 ± 13.82</td>
<td>0.67 ± 0.28</td>
<td>12.9 ± 5.13</td>
<td>1.89 ± 1.22</td>
</tr>
<tr>
<td>F2</td>
<td>0.5%</td>
<td>1:2</td>
<td>3:1</td>
<td>23.29 ± 0.77</td>
<td>1056.33 ± 31.3</td>
<td>0.65 ± 0.18</td>
<td>15.2 ± 4.17</td>
<td>2.329 ± 2.20</td>
</tr>
<tr>
<td>F3</td>
<td>0.5%</td>
<td>1:1</td>
<td>5:1</td>
<td>20.97 ± 2.53</td>
<td>614.23 ± 11.36</td>
<td>0.55 ± 0.16</td>
<td>17.35 ± 3.5</td>
<td>2.438 ± 0.78</td>
</tr>
<tr>
<td>F4</td>
<td>0.5%</td>
<td>1:2</td>
<td>5:1</td>
<td>36.10 ± 2.20</td>
<td>755.8 ± 6.73</td>
<td>0.34 ± 0.45</td>
<td>22.75 ± 3.5</td>
<td>3.682 ± 0.56</td>
</tr>
<tr>
<td>F5</td>
<td>0.75%</td>
<td>1:1</td>
<td>3:1</td>
<td>25.23 ± 1.89</td>
<td>1097.6 ± 32.29</td>
<td>0.55 ± 0.63</td>
<td>18.4 ± 3.9</td>
<td>3.989 ± 3.2</td>
</tr>
<tr>
<td>F6</td>
<td>0.75%</td>
<td>1:2</td>
<td>3:1</td>
<td>48.71 ± 1.15</td>
<td>1168.33 ± 28.4</td>
<td>0.29 ± 0.17</td>
<td>23.1 ± 2.59</td>
<td>4.199 ± 2.29</td>
</tr>
<tr>
<td>F7</td>
<td>0.75%</td>
<td>1:1</td>
<td>5:1</td>
<td>72.15 ± 0.94</td>
<td>656.1 ± 5.71</td>
<td>0.45 ± 0.13</td>
<td>20.1 ± 3.3</td>
<td>5.678 ± 0.56</td>
</tr>
<tr>
<td>F8</td>
<td>0.75%</td>
<td>1:2</td>
<td>5:1</td>
<td>85.15 ± 1.41</td>
<td>778.07 ± 11.22</td>
<td>0.16 ± 0.26</td>
<td>25.2 ± 2.63</td>
<td>5.893 ± 0.98</td>
</tr>
</tbody>
</table>

Fig. 2. Plots for the main effect of different factors according to $2^3$ factorial design on the entrapment efficiency: [A] chitosan conc. %, [B] drug to polymer ratio, [C] chitosan to TPP ratio.
(P < 0.0001) which was declared by Leelapornpisid [8], flocculation of nanoparticles happened due to the presence of excess anions by using 3:1 chitosan to TPP ratio leading to instability of the system hence decreased zeta potential. Also the same result was reported by Gan et al. [35], Stoica and Ion [31] and Ing et al. [36] who found sharp decrease in zeta potential when decreasing chitosan to TPP ratio. Fig. [4] illustrated the effect of the different factors on the zeta potential of the system.

4.1.5. In-vitro release study

The release pattern was studied in pH 5.5 phosphate buffer. Release profile of diphenhydramine hydrochloride from the prepared nanoparticles is shown in (Fig. 5). The prepared nanoparticles could prolong the drug release when compared to drug powder. About 20–40% of diphenhydramine hydrochloride were released from the prepared nanoparticles after 15 min in comparison to diphenhydramine hydrochloride powder (100% were released after 15 min) as shown in Figs. [5A] and [5B]. The prepared nanoparticles were able to prolong the drug release up to 6hrs. An initial burst release of diphenhydramine hydrochloride occurred due to desorption of diphenhydramine hydrochloride molecules from the surface of the particles, with 20–40% of the drug being released within 15 min from the beginning of the experiment. This was in concordance with the work of Silva et al. [32] and Patel et al. [37].

Increasing the chitosan concentration from 0.5% to 0.75% led to a prolongation of release of diphenhydramine hydrochloride from

Fig. 3. Plots for the main effect of different factors according to $2^3$ factorial design on the particle size: [A] chitosan conc. %, [B] drug to polymer ratio, [C] chitosan to TPP ratio.
Fig. 4. Plots for the main effect of different factors according to $2^3$ factorial design on the zeta potential: [A] chitosan conc. %, [B] drug to polymer ratio, [C] chitosan to TPP ratio.

Fig. 5A. Release pattern of diphenhydramine hydrochloride from chitosan nanoparticles at pH 5.5.

Fig. 5B. Release pattern of diphenhydramine hydrochloride from chitosan nanoparticles at pH 5.5.
chitosan nanoparticles where 95% of the drug was released after 6 h when compared to the powder drug100%. Also the initial burst release was reduced. Similar results were reported by Gan and Wang [38]. The increased viscosity of colloidal system at higher chitosan concentration allowed the formation of denser chitosan–diphenhydramine hydrochloride particles upon interaction with sodium tripolyphosphate, resulting in greater crosslink density with less swelling ability, and thereafter reduced diphenhydramine hydrochloride initial release [39]. Concerning drug to polymer ratio, by increasing the drug to polymer ratio from 1:1 to 1:2, it was found that the release of the drug decreased. This may be due to by increasing the drug to polymer ratio, the amount of chitosan increased, forming firm wall particles allowing the slow release of the drug from the nanoparticles to the external media.

Regarding the chitosan to TPP(w/w) ratio, by increasing the ratio from 3:1 to 5:1, the release of diphenhydramine hydrochloride from the diphenhydramine hydrochloride loaded chitosan nanoparticles decreased and this could be attributed to the formation of more stable nanoparticles when the ratio 5:1 was used in chitosan nanoparticles the preparation: These results were in accordance with Ing.et al. [36] and Gan and wang [38].

### Table 4

Coefficient of determination (R2) of diphenhydramine hydrochloride release data from the prepared formulae according to zero order, first order and Higuchi Model.

<table>
<thead>
<tr>
<th>Formula code</th>
<th>Zero order kinetics</th>
<th>First order kinetics</th>
<th>Higuchi diffusion model</th>
<th>Mechanism of release</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>0.8322</td>
<td>0.9799</td>
<td>0.9253</td>
<td>First order</td>
</tr>
<tr>
<td>F2</td>
<td>0.9034</td>
<td>0.9718</td>
<td>0.9725</td>
<td>Higuchi model</td>
</tr>
<tr>
<td>F3</td>
<td>0.9401</td>
<td>0.8752</td>
<td>0.9917</td>
<td>Higuchi model</td>
</tr>
<tr>
<td>F4</td>
<td>0.8638</td>
<td>0.9673</td>
<td>0.9575</td>
<td>First order</td>
</tr>
<tr>
<td>F5</td>
<td>0.8569</td>
<td>0.9696</td>
<td>0.9559</td>
<td>First order</td>
</tr>
<tr>
<td>F6</td>
<td>0.8993</td>
<td>0.9397</td>
<td>0.9773</td>
<td>Higuchi model</td>
</tr>
<tr>
<td>F7</td>
<td>0.881</td>
<td>0.9327</td>
<td>0.9634</td>
<td>Higuchi model</td>
</tr>
<tr>
<td>F8</td>
<td>0.8876</td>
<td>0.9272</td>
<td>0.9694</td>
<td>Higuchi model</td>
</tr>
</tbody>
</table>

4.1.5.1. Kinetic analysis. Kinetic analysis of the release profiles of the prepared nanoparticles are shown in Table (4). According to the values of coefficient of determination (R²) the mechanism of the drug release

![Fig. 6. DSC thermograms of: [A] plain nanoparticles, [B] diphenhydramine hydrochloride powder and [C] diphenhydramine hydrochloride loaded chitosan nanoparticles [F8].](image)
was defined. T90% of drug release for the different formulations was calculated and are shown in Table [3] Most of formulae showed best fitting to higuchi model suggesting that the drug release is governed by diffusion release mechanism, whereas the others fitted best to the first order model which may suggest the dissolution mechanism of the drug due to changes in the structure of the nanoparticles [40]. In diffusion, controlled mechanism, according to higuchi equation, pseudo-steady state is maintained during drug release [41]. The highest T90% of drug release was achieved by formula F8 (5.893 h ± 0.98) indicating prolonged drug release.

4.1.6. Differential scanning calorimetry (DSC)

Thermograms of plain chitosan nanoparticles, diphenhydramine hydrochloride powder and diphenhydramine hydrochloride loaded chitosan nanoparticles [F8] were shown in Fig. [6]. Plain chitosan nanoparticles showed a peak at 92.27 °C [A]. Diphenhydramine hydrochloride powder showed a sharp peak at 170.43 °C [B], corresponding to its melting temperature [11]. Diphenhydramine hydrochloride loaded chitosan nanoparticles showed a mild pre shift peak from 92.27 °C to 71.85 °C [34]. The peak position of diphenhydramine hydrochloride thermogram disappeared in the chitosan loaded nanoparticles thermogram, suggesting the loss of crystallinity of the drug in nanoparticles which could enhance the entrapment efficiency of the drug [42,43].

4.1.7. X-ray diffraction (XRD)

XRD analysis provides the crystal lattice arrangements and gives the information regarding the degree of crystallinity in the formulation. It is also used for the identification of physical state of drug in the formulation [37]. X-ray diffraction pattern of plain chitosan nanoparticles [A], diphenhydramine hydrochloride powder [B] and diphenhydramine hydrochloride loaded chitosan nanoparticles [F8] [C] were illustrated in Fig. [7]. The plain chitosan nanoparticles depicted an amorphous character, while diphenhydramine hydrochloride powder depicted sharp crystalline peaks in range of 2θ = 0–30° which nearly disappeared in the entrapped system, except a small peak at 2θ = 20°. Therefore, it is an evident that there is a significant loss of crystallinity of the drug while being entrapped in the polymeric system.

4.1.8. In- vivo evaluation

The preliminary study was done for the determination of the histamine dose to produce a typical wheal. The dose 200 mg/ml resulted in appearance of a typical wheal with area 150.67 mm² ± 10.2% which was used in the experiment.

The optimized formula selected by the Design Expert software according to the defined constraints for each independent variable for the prepared nanoparticles with a desirability of 0.926 was F8 (0.375% of diphenhydramine hydrochloride, drug to polymer 1:2, chitosan concentration 0.75% and chitosan to TPP (w/w) 5:1), showed the highest % entrapment efficiency 85.15% ± 1.41, suitable particle size 778.07 nm ± 11.22, the smallest PDI 0.16 ± 0.26, the highest zeta potential 12.83 mV ± 0.98. This formula showed the highest percentage wheal suppression after 2 h (Fig. 10). The histopatological observations showed no histological and pathological changes in skin treated with the optimized formula (Fig. 8).
potential 25.2 mV ± 2.63, and the highest T 90% 5.89 h ± 0.98 was selected as candidate formula for in-vivo study.

4.1.8.1. Skin sensitivity studies and histopathological examination

After the application of the candidate formula F8 on the dorsal skin of the rats, no visual changes were observed and no pathological changes were found on the treated skin as shown in Fig. [8]. Thus, the results confirmed the dermal safety of the prepared formula on the rat skin.

4.1.8.2. Evaluation of antihistaminic activity

4.1.8.2.1. In-vivo pharmacodynamic evaluation

After topical application of both, the candidate formula (F8) and the marketed gel, on the inflamed rat skin in each group, the mean wheal areas at each time interval were calculated and illustrated graphically in Fig. [9], also the mean percentage suppression of histamine induced wheal area was also calculated and represented in Fig. [10].

Upon application of F8 on the rat skin the wheal area decreased after 2 h by about 71% from 150 mm² ± 7.8 (the initial wheal area) to 43.6 mm² ± 4.9, while for the marketed gel the wheal area decreased from 155 mm² ± 6.1–82.1 mm² ± 8.54 with about 47% decrease.

4.1.8.2.2. Histopathological examination

4.1.8.2.2.1. Histological structure of the intact rat skin layers

Fig. [8A] showed normal epidermis (black arrow head), covered by irregular non cellular scaly keratin (black arrow). Normal papillary dermis with slightly edema [P]; intact hair follicle (yellow arrow head), and few number of eosinophils (green arrow) were noticed. [400X, H&E]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4.1.8.2.2.2. Histological structure of the inflamed rat skin layers

As shown in Figs. [11A] and [11B], when histamine was injected intradermally, a spontaneous flare and swollen spherical area was obtained and was clearly detected visually. Histological structure of rat skin revealed an increased skin thickness due to edema but less than the histamine group (double head black arrow), normal epidermis (black arrow head), covered by irregular non cellular scaly keratin (black arrow). Edematous papillary dermis [P] with edematous reticular dermal [D] layers were observed but less than the histamine group; dilated congested blood vessels (green arrow) were deep in the edematous hypodermis [H], multiple hair follicles (yellow arrow head), and sebaceous glands (yellow arrow) were noticed in the dermis. [400X, H&E]. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4.1.8.2.2.3. Histological structure of the treated rat skin

The histopathological patterns of the inflamed and treated skin varied greatly according to the applied formula. After application of the candidate formula (F8), as shown in Fig. [12], mild inflammation compared to marketed gel treated groups was observed with reduced skin edema near to the normal control group and reduced edema level.
in dermis and hypodermis were noticed. Few number of the inflammatory cell infiltrates were seen. There was no congestion or dilatation in the blood vessels in the hypodermis.

After application of the marketed gel, as shown in Fig. [13], moderate inflammation compared to nanoparticles treated groups, with increased skin thickness due to edematous changes but less than the histamine group and more than the normal control group. Papillary and reticular dermis showed moderate edema also severe edema in the hypodermis with congested dilated blood vessels were seen.

Fig. [14] showed high correlation [r = 0.97704] between the in-vitro release study of diphenhydramine hydrochloride from the chitosan nanoparticles and the in-vivo wheal reduction response. These findings might suggest that the prepared diphenhydramine hydrochloride loaded chitosan nanoparticles F8 were targeted to the epidermis and were able to decrease the skin allergy induced by histamine injection with a lower concentration of diphenhydramine hydrochloride and better efficacy when compared to the marketed gel.

5. Conclusion

It was concluded that diphenhydramine hydrochloride loaded chitosan nanoparticles were successfully prepared by ionic gelation technique. The antihistaminic activity was significantly enhanced by using chitosan nanoparticles. F8 showed the highest entrapment efficiency (85.15% ± 1.41), zeta potential (25.2 mV ± 2.63) and T90% of drug release (5.893 h ± 0.98) with adequate particle size (778.07 nm ± 11.22) and the lowest PDI (0.16 ± 0.26). The Kinetic release model was according to higuchi model indicating the drug release was governed by diffusion release mechanism. Skin irritation test and histopathological studies on rat skin revealed the safety of the formulation for dermal use with reduction in the wheal area after 2 h representing 71% decrease from the initial wheal area. The anti-histamine activity of the drug was significantly increased (p < 0.05) when diphenhydramine hydrochloride loaded chitosan nanoparticles was used. From the findings of the study, the developed formula could have a potential for topical delivery of diphenhydramine hydrochloride with increased patient compliance. This promising nanoparticulate system contains an adequate amount of drug sufficient to reduce the allergic reactions in comparison to the conventional gel of diphenhydramine hydrochloride which requires multiple applications to reach to all allergic reactions in comparison to the conventional gel of diphenhydramine hydrochloride which requires multiple applications to reach to therapeutic concentration.

Declaration of competing interest

The authors have no conflict of interest to declare.

References


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