ABSTRACT

Objective: This study aims to report the optimum formula for BSA nanoparticles cholecalciferol (BSA-NP cholecalciferol), which can increase the solubility of cholecalciferol.

Methods: BSA cholecalciferol nanoparticles were prepared by desolvation method with variations in solvent/non-solvent ratio, BSA concentration, pH of BSA solution, and cholecalciferol concentration. For this purpose, particle size, polydispersity index, and zeta potential were measured. Furthermore, the solubility test of the best BSA-NP's cholecalciferol formula was carried out.

Results: The most optimal BSA nanoparticle cholecalciferol characterization results have a particle size of 166.6±50.3 nm, a zeta potential of -32.1 mV, and a percentage encapsulation efficiency (%EE) for cholecalciferol of 82.9±0.72%. The solubility of BSA-NP cholecalciferol is four times higher than that of pure cholecalciferol.

Conclusion: The optimum formula for BSA-NP cholecalciferol with a solvent/non-solvent ratio of 1/2, a concentration of BSA of 2.5%, a BSA solution pH 6, and a cholecalciferol concentration of 0.1% will increase the solubility of cholecalciferol by four times compared to pure cholecalciferol.

Keywords: BSA, Cholecalciferol, Nanoparticle, Desolvation, Solubility

INTRODUCTION

Based on research in the last ten years shows that Vitamin D and its metabolites have activity in preventing or treating various cancers, one of which is breast cancer. This is a new role for vitamin D that differs from its traditional role in controlling calcium and bone metabolism [1].

Vitamin D is a secosteroid hormone that can be classified into two main different forms, namely vitamin D₃ (ergocalciferol) and vitamin D₅ (cholecalciferol). Cholecalciferol is naturally synthesized in the skin of humans and animals through exposure to sunlight [2]. Cholecalciferol has physicochemical properties, including being a hydrophobic compound that cannot be dispersed in liquid media, having low solubility, low bioavailability, and high toxicity [3], as well as a short half-life in the bloodstream [4], and drug release occurs before it reaches the body's target cells [5]. One of the most common problems in pharmacological solubility. To ensure that the drug is absorbed and has a therapeutic impact, its solubility will be correlated with the rate of absorption [6]. Efforts to overcome the limitations of cholecalciferol require the development of an appropriate delivery system so that the drug can reach the target cell. The effective size of nanoparticles to enter cancer cells by increasing their permeability and retention is less than 200 nm [7, 8]. Based on research conducted by Almouazen et al. (2013), it is known that nanoencapsulation is a more suitable system for cholecalciferol than the nanosphere system, where nanoencapsulation has a better percentage of encapsulation efficiency (92%) compared to the nanosphere system (±69.8%) [5].

Various carrier systems are available for the encapsulation process of a drug that is problematic both in formulation and delivery. In this study, a protein was chosen, namely serum albumin (Bovine Serum Albumin, or BSA). The BSA will be synthesized as a carrier for nanoparticles called BSA-NP. BSA is a non-toxic, biodegradable polymer that is stable against changes in pH (4–9), organic solvents, and at 60 °C for 10 h [9].

BSA is inert, biocompatible, relatively inexpensive, can form nanoparticles with various techniques, and most importantly, can penetrate better and more deeply into cancer cells [10]. In addition, the basic consideration for selecting BSA as a carrier system is that BSA is known as a carrier for various molecules, one of which is vitamin D [11].

Albumin nanoparticles can be prepared using several techniques, namely desolvation, emulsification, thermal gelation, nano-spray drying, nanoparticle albumin-bound technology (Nab-Technology), and self-assembly methods. This study used the desolvation technique because several studies reported the successful encapsulation of hydrophobic drugs into polymer nanoparticles using this technique [10, 12]. The principle of this technique is to change the solubility of BSA in water by adding a desolvation factor [13]. Various parameters that can affect the formation of BSA-NPs with hydrophobic drugs using desolvation techniques have been extensively studied [10]. This research aimed to explore desolvation conditions to produce BSA nanoparticles loaded with cholecalciferol with physical and chemical characterization results that meet specifications and increase the solubility of cholecalciferol in water.

MATERIALS AND METHODS

Materials

The materials used in this study were Cholecalciferol (Sigma Aldrich), BSA (Sigma Aldrich), Ethanol absolute (Merck), mill-Q water, and Glutaraldehyde (Merck).

Preparation of BSA-NP cholecalciferol

Preparation of BSA-NP Cholecalciferol using a bottom-up system with a modified desolvation technique the initial stage of this...
synthesis is to dissolve BSA in milli-Q water. The preparation was stirred continuously until completely dissolved using a magnetic stirrer at 1000 rpm at room temperature (20–25 °C) for 10 min. Cholecalciferol is dissolved in ethanol until dissolved. Furthermore, the cholecalciferol solution was added to the BSA solution using a microtube pump at a speed of 2 ml/min, accompanied by constant stirring using a magnetic stirrer at 1000 rpm at room temperature (20–25 °C) until a cloudy solution was formed. Then 235 µl glutaraldehyde (8% v/v) as a crosslinker was added. The reaction was stabilized by stirring using a magnetic stirrer at 1000 rpm for 24 h. The resulting nanoparticles were then purified by centrifugation three times at 10000 rpm for 30 min, and then the formed pellets were dispersed again with milli-Q water and sonicated for 60 min at room temperature (20–25 °C). The resulting solution was stored at -80 °C overnight, then freeze-dried and stored at 4 °C for further analysis [5, 14]. In this study, optimization was carried out by varying the ratio of the amount of solvent to non-solvent, the concentration of BSA, the pH of the BSA solution, and the concentration of cholecalciferol.

Characterization of prepared BSA-NPs cholecalciferol

Particle size, polydispersity index (PDI), and zeta potential analysis

Particle size, PDI, and zeta potential of the prepared nanoparticles were analyzed by a dynamic light scattering (DLS) technique analyzer. The sample was analyzed using Horiba 5z-100 using aqueous media with 10 times sample dilution at room temperature at 25 °C [15, 16]. Each sample was analyzed in triplicate as mean±SD [17, 18].

Encapsulation efficiency (%EE)

Encapsulation efficiency (EE) was determined by measuring the unencapsulated cholecalciferol by centrifugation. Several hundred cholecalciferol was dispersed in methanol and centrifuged at 1000 rpm at 4 °C for 15 min. Cholecalciferol, which was not encapsulated in the supernatant, was measured using a spectrophotometer at a wavelength of 264.6 nm [19]. %EE is calculated using the following formula:

%EE = \frac{\text{Amount of drug encapsulated}}{\text{Amount of drug initially taken}} \times 100

The analysis was done in triplicate; the results were expressed mean±SD.

Morphology studies

The morphology of BSA-NPs Cholecalciferol was examined by scanning electron microscopy (SEM) (Model JEOL, JSMS660 system). The samples were placed into the stub and coated with platinum. BSA-NP Cholecalciferol photomicrographs were taken at 20 K with 50000 magnifications [19].

Solubility test

The solubility test of cholecalciferol and BSA-NP cholecalciferol was carried out by placing each sample in distilled water at pH 7.4 and stirring constantly for 24 h. The solution was filtered and diluted to a ratio of 1:3. The concentration of cholecalciferol in the solution was analyzed using UV-Vis spectrophotometry at a wavelength of 264.6 nm [20].

RESULTS AND DISCUSSION

Effects of ratio solvent/non-solvent

Based on the results of the optimization carried out with the parameter of the volume ratio of solvent/non-solvent (1/2), namely the volume of 10 ml of BSA solution and 20 ml of ethanol solution. These results indicate that the optimum conditions for the parameters of particle size, polydispersity index, and zeta potential can be achieved when the amount of ethanol solution is twice the amount of BSA solution. This is in previous research by Tanhini et al. (2018), who stated that the formation of nanoparticles depends on the volume of non-solvent added; if the non-solvent volume is insufficient for the solute to reach saturation, precipitation will not occur or will produce broken particles with a wide size distribution [10]. In addition, a high solvent/non-solvent ratio can cause an increase in particle size and polydispersity index. This is by previous research by Jahanshasi et al. (2008), who stated that samples can be highly polydisperse, resulting in inaccurate measurements at high solvent/non-solvent ratios [21].

Effects of BSA concentration

Based on the results of the optimization carried out with the BSA concentration parameter, it is known that the best formula is the one with a BSA concentration of 2.5%. BSA concentration greatly affects the results of the physical characterization of BSA-NP cholecalciferol, which can be explained through the nucleation theory. In this theory, it is explained that with increasing BSA concentration, the viscosity will increase and the frequency of protein transport between water and the desolvent agent will decrease, causing slower nucleation rates and resulting in larger particle sizes [22]. The results obtained in this study are different from previous research conducted by Noorani L. et al. who explained that the higher the concentration of BSA, the greater the possibility of coagulation through electrostatic and hydrophobic interactions [23]. In this study, the results showed that the higher the concentration of BSA, the smaller the particle size. This can occur due to other factors such as the pH of the BSA solution or the amount of non-solvent that affects the protein deposition process by changing the environment and changing its conformation [10].

Effects of pH BSA solution

Based on the optimization results carried out with the pH parameters of the BSA solution, it is known that the best formula is the formula with BSA solution pH 6. The pH value of the BSA solution is an important factor that will coagulate BSA during the desolvent process because the chemical properties and structure of protein molecules are strongly influenced by changes in pH [24]. Changes in the pH value of the BSA solution will significantly change the net charge on the protein surface because it is composed of hydrogen, amino acids that interact with ions in the solution. The isoelectric point (pI) of BSA is at 4.9 and at this pH, the surface charge of the protein is zero. At pH 4.9, the electrostatic repulsion is less so that amorphous aggregates will easily form through non-specific interactions, especially those that are hydrophobic, causing large particle sizes [25]. Conversely, when the pH of the solution moves away from the BSA pI value, the particle diameter will decrease because positive charges can be generated by protonation of primary amino groups (especially lysine), while negative charges are generated by deprotonation of carboxylic acid groups (glutamate and aspartate). The resulting charge across the protein surface causes greater electrostatic repulsion between molecules and a decrease in hydrophobic interactions, thereby reducing aggregation and favoring structural reorganization of the protein, which can significantly alter the interaction and/or binding of drug compounds [26].

Effects of cholecalciferol concentration

Based on the optimization results carried out with the cholecalciferol concentration parameter, it is known that the best formula is a formula with a cholecalciferol concentration of 0.1% where the formula meets the desired physicochemical characteristics criteria, namely particle size ≤200 nm, polydispersity index ≤0.7 and zeta potential >30 mV or <30 mV. The drug concentration in the initial solution is also important in the particle formation process [27]. This drug concentration effect is associated with an increase in dissolved drug concentration, which will increase the viscosity, which will inhibit diffusion between the solvent and the anti-solvent, causing non-uniform saturation, slower nucleation rates, and increased particle agglomeration resulting in larger particles [28, 29]. This is in research conducted by Aliabali et al., where it is known that the higher the drug concentration, the larger the particle size [30]. The optimization results for the four parameters can be seen in Table 1.
Table 1: Size, zeta potential, and polydispersity index (PDI) of BSA-NP cholecalciferol the effect of the various experimental parameters used in this work

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Particle size (nm)</th>
<th>Zeta potential (mV)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent/Non-Solvent Volume</td>
<td>1/3</td>
<td>272.7±117.6</td>
<td>-32.9</td>
</tr>
<tr>
<td>Ratio</td>
<td>2/5</td>
<td>313.4±152.9</td>
<td>-33.7</td>
</tr>
<tr>
<td></td>
<td>1/2</td>
<td>241.8±57.3</td>
<td>-33.5</td>
</tr>
<tr>
<td></td>
<td>2/3</td>
<td>350.0±104.4</td>
<td>-31.7</td>
</tr>
<tr>
<td></td>
<td>1/1</td>
<td>298.4±138.3</td>
<td>-26.1</td>
</tr>
<tr>
<td>BSA concentration</td>
<td>1.0%</td>
<td>200.0±140.7</td>
<td>-30.7</td>
</tr>
<tr>
<td></td>
<td>1.5%</td>
<td>149.2±66</td>
<td>-25.5</td>
</tr>
<tr>
<td></td>
<td>2.0%</td>
<td>143.5±66.3</td>
<td>-26.4</td>
</tr>
<tr>
<td></td>
<td>2.5%</td>
<td>137.3±55.7</td>
<td>-31.5</td>
</tr>
<tr>
<td></td>
<td>3.0%</td>
<td>124.4±78.8</td>
<td>-22.4</td>
</tr>
<tr>
<td>BSA solution pH</td>
<td>2</td>
<td>2369±208.7</td>
<td>-27.9</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>150.8±76.4</td>
<td>-23.2</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>137.3±55.7</td>
<td>-31.5</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>2797±386</td>
<td>-29.9</td>
</tr>
<tr>
<td>Cholecalciferol concentration</td>
<td>0.25%</td>
<td>268.0±85.6</td>
<td>-35.2</td>
</tr>
<tr>
<td></td>
<td>0.20%</td>
<td>255.0±68.8</td>
<td>-47.1</td>
</tr>
<tr>
<td></td>
<td>0.15%</td>
<td>179.2±58.3</td>
<td>-41.2</td>
</tr>
<tr>
<td></td>
<td>0.10%</td>
<td>160.0±75.1</td>
<td>-34.9</td>
</tr>
<tr>
<td></td>
<td>0.05%</td>
<td>158.0±65.6</td>
<td>-41.8</td>
</tr>
<tr>
<td></td>
<td>0.025%</td>
<td>137.3±55.7</td>
<td>-31.5</td>
</tr>
</tbody>
</table>

*Data are expressed as means±SD, n=3

Characterization of BSA-NP cholecalciferol freeze-drying results

After obtaining the optimum formula, a freeze-drying process was carried out to obtain BSA-NPs Cholecalciferol in powder form. The product resulting from the freeze drying will be subjected to further tests consisting of a Particle Size Analyzer-Zeta sizer (PSA-Zeta sizer) to find out particle size, polydispersity index (PI), zeta potential, %EE, Scanning Electron Microscopy (SEM) to determine the shape of the surface morphology, Differential Scanning Calorimetry (DSC) to determine the thermal properties, and X-ray Diffractometry (XRD) to determine the crystallinity [31]. Particle size, PDI, zeta potential, and %EE test results for freeze-dried products can be seen in table 2.

Table 2: Particle size characterization results, PDI, zeta potential, and %EE of BSA-NP cholecalciferol

<table>
<thead>
<tr>
<th>Formulas</th>
<th>Physicochemical characterization</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
<th>%EE*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Freeze-drying</td>
<td>156.1±46.4</td>
<td>0.584</td>
<td>-37.3</td>
<td>87.28±0.130</td>
</tr>
<tr>
<td>After Freeze-drying</td>
<td>166.6±50.3</td>
<td>0.825</td>
<td>-32.1</td>
<td>82.90±0.720</td>
</tr>
</tbody>
</table>

*Data are expressed as means±SD, n=3

Based on the data contained in table 2, it can be seen that the particle size of BSA-NP Cholecalciferol before and after freeze-drying is not much different and still meets the desired criteria, namely a particle size of ±200 nm. There was an increase in the polydispersity index parameter from 0.584 to 0.825. The increase in the polydispersity index is because, during freeze drying, particle aggregation occurs, resulting in large particle sizes and a wider distribution of particle sizes [32]. Following previous research by Araya-Sibaja AM et al, which states that if the PI value is <0.3, the particle size shows a narrow distribution, and if the PI value is >0.3, the particle size is considered to have a wide distribution [33]. The zeta potential results from BSA-NP Cholecalciferol both before and after freeze-drying meet the desired criteria. This is by research conducted by Pal et al, who found that if the zeta potential value is less than -30 mV or more than +30 mV, it tends to be stable and the particles will not flocculate during the storage period [34]. In terms of chemical characteristics, namely % encapsulation efficiency, it shows that BSA-NP Cholecalciferol before freeze drying is of greater value than BSA-NP Cholecalciferol after freeze drying, but the %EE values of the two products still meet the expected criteria, namely >70% [35].

Scanning electron microscopy (SEM)

SEM analysis was performed on BSA-NP Cholecalciferol to determine the morphology and form of the resulting nanoparticles. The results of the morphological analysis can be seen in fig. 1.

![Fig. 1: SEM Image of BSA-NP cholecalciferol](image-url)
Based on the results shown in fig. 1, it can be seen that the morphology of BSA-NP cholecalciferol shows a rounded and smooth surface, monodisperse, and smooth texture. This is by previous research stated that the morphology of albumin nanoparticles and a drug with albumin nanoparticle carriers is spherical [30, 36].

**Solubility test of BSA-NP cholecalciferol**

Drug solubility in water is one of the important physicochemical factors for drug absorption. In addition, good aqueous solubility has been reported to play a major role in enhancing the pharmacokinetic properties of drugs, including bioavailability and therapeutic effects [37]. The water solubility test results for BSA-NP cholecalciferol compared to pure cholecalciferol can be seen in fig. 2.

Based on the data in fig. 2, the solubility of pure cholecalciferol in water is 0.29±0.012 µg/ml while the solubility of BSA-NPs cholecalciferol in water is 1.17±0.012 µg/ml. These results indicate that the solubility of BSA-NPs cholecalciferol is four times higher when compared to its pure form of cholecalciferol.

![Fig. 2: Solubility test of cholecalciferol and BSA-NP cholecalciferol, all values shown in graph are measured as mean±SD, n=3](image)

**CONCLUSION**

Cholecalciferol was prepared in the bovine serum albumin (BSA) nanoparticle system using the desolvation method and optimum conditions of a solvent/non-solvent ratio of 1/2, concentration of BSA of 2.5%, BSA solution pH of 6 and cholecalciferol concentration of 0.1% will increase its solubility is four times higher than its pure form of cholecalciferol.

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**AUTHORS CONTRIBUTIONS**

All the authors have contributed equally.

**CONFLICT OF INTERESTS**

The authors have no conflict of interest to declare.

**REFERENCES**


