DEVELOPMENT OF GAMMA ORYZANOL-LOADED NANOSTRUCTURED LIPID CARRIERS BY USING GLYCERYL STEARATE BLENDED PEG-100 STEARATE AS SOLID LIPID AND COLD PRESSED RICE BRAN OIL AS LIQUID LIPID

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Abstract: In this study, γ-oryzanol-loaded nanostructured lipid carriers (NLCs) were developed by using Tego® Care 165 (glyceryl stearate combined PEG-100 stearate, 50:50 (w/w)) as solid lipid and cold pressed rice bran oil as liquid lipid. They were varied by a ratio of solid lipid to liquid lipid and the amount of total lipids. The results showed instability in all formulations, except γ-NLC1 and γ-NLC9. The particle sizes of γ-NLC1 and γ-NLC9 showed smaller than 200 nm and polydispersity index values were less than 0.2. Zeta potential values were higher than -20 mV. Both formulations showed higher entrapment efficiency than 99%. Increasing the content of lipid showed precipitation from excess lipid. The mixed of three nonionic surfactants could stabilize formulations better than the mixed of two nonionic surfactants. Interestingly, particle sizes of NLCs reduced when the amount of nonionic surfactant increased. In summary, γ-NLC9 showed good physicochemical properties and promising stable formulation. Moreover, Tego® Care 165 and cold pressed rice bran oil are able to use solid lipid and liquid lipid for γ-oryzanol encapsulation.

Keywords: γ-oryzanol, nanostructured lipid carriers, cold pressed rice bran oil

บทคัดย่อ: ในการศึกษานี้ตัวพาระบบไขมันขนาดนาโนเมตรบรรจุแกมมาออริซานอลจะถูกพัฒนาโดยใช้เทโกแคร์165 (กลีเซอริลสเตียเรทผสมอยู่กับโพลีเอธิลีนไกลคอล-100สเตียเรทในอัตราส่วน50:50โดยน้ำหนัก) เป็นไขมันแข็งและน้ำมันรำข้าวบีบเย็นเป็นไขมันเหลว ทำให้เกิดการเปลี่ยนแปลงอัตราส่วนของไขมันแข็งและไขมันเหลวในตัวพาระบบ ระบบไขมันขนาดนาโนเมตร จากผลการทดลองพบว่าทุกสูตรตัวรับนั้นมีความไม่คงตัวยกเว้น γ-NLC1 และ γ-NLC9 โดยขนาดอนุภาคของตัวรับนั้นมีขนาดเล็กกว่า 200 นิวเคลียร์ มีค่าดัชนีการกระจายตัวของอนุภาค (PI) ต่ำกว่า 0.2 และค่าความต่างศักย์ไฟฟ้าอยู่ที่ -20 มิลลิโวลต์ ซึ่งสูตรสูตรตัวรับสามารถกักเก็บสารได้มากกว่า 99% จากการทดลองพบว่าการใช้ปริมาณไขมันเพิ่มขึ้นในระบบส่งผลให้เกิดการตกตะกอน ซึ่งอาจจะเกิดจากการถูกไขมันแข็งเกินไป การใช้สารลดแรงตึงผิวที่ไม่มีประจุชนิดสามชนิดร่วมกัน พบว่าทำให้ตัวรับมีความคงตัวมากกว่าการใช้สารลดแรงตึงผิวที่ไม่มีประจุชนิดสองชนิดร่วมกัน สูตรสูตรที่มีค่าดัชนีการกระจายตัวอยู่ที่ PI ต่ำกว่า 0.2 ตรงกับผลการทดลองที่ส่งผลให้ตัวรับมีความคงตัวมากกว่า 99% และมีค่าความต่างศักย์ไฟฟ้าอยู่ที่ -20 มิลลิโวลต์ สูตรนี้ได้รับการยอมรับเป็นสูตรตัวรับที่ดีที่สุด ทั้งในกระบวนการกักเก็บสารและที่มีคุณสมบัติทางเคมีฟิสิกส์ที่ดีที่สุดและมีคุณสมบัติทางฟิสิกส์ที่ดีที่สุด นอกจากนี้แทกโย Care 165 และน้ำมันรำข้าวบีบเย็นสามารถนำมาใช้เป็นไขมันแข็งและไขมันเหลวสำหรับการกักเก็บแกมมาออริซานอลได้อย่างมีประสิทธิภาพ

คำสำคัญ: แกมมาออริซานอล, ตัวพาระบบไขมันขนาดนาโนเมตร, น้ำมันรำข้าวบีบเย็น
INTRODUCTION

Nanostructured lipid carriers (NLCs) are the second generation of solid lipid nanoparticles (SLNs). SLNs have some problems such as drug encapsulation limitation and drug expulsion. Because they consist of only solid lipid and after preparation at least a part of particles crystallizes in a higher energy modification (α or β). During storage, these modifications can transform to lower energy, more ordered β modification. Due to its high degree of order, the number of imperfections in the crystal lattice is reduced and leading to limited encapsulation and drug expulsion (Pardieke et al., 2009). In contrast, NLCs are composed of solid lipid and liquid lipid. Liquid lipid in NLCs can decrease the order of crystalline structure and increase solubility of drug in lipid structure which affect to more imperfections in the crystal lattice (Varshosaz et al., 2010; Rahman et al., 2013). Thus, they have more lipid matrix for increasing drug loading capacity and higher entrapment efficiency. NLCs may improve physical and chemical stability of drug, drug loading capacity, entrapment efficiency of drug and including controlled drug release better than SLNs. Moreover, NLCs are used for topical delivery because they not only increase skin hydration effect but also very effective permeate through the skin (Müller et al., 2002; Pardeike et al., 2009; Pardeike, et al., 2010). Interestingly, lipid nanoparticles system can increase efficiency and stability of antioxidant compound and chemical sunscreen (Xia et al., 2010; Chen et al., 2013). Therefore, NLCs as the new drug delivery system have high potential for skin topical and transdermal application.

It has been reported that SLNs containing ɤ-oryzanol occurred gelation and limitation of entrapment efficiency (Seetapan et al., 2010). Therefore, NLCs are developed to solve some problems of SLNs. Mostly composition of liquid lipid in NLCs is synthetic oil or processed natural oil but not in cold pressed oil such as rice bran oil. Interestingly, the highest fatty acid composition in cold pressed rice bran oil is oleic acid, which is used as liquid lipid in NLCs formulation (Yuan et al., 2007; Yoshie et al., 2009). Moreover, cold pressed rice bran oil is a good solvent of ɤ-oryzanol thus it is suitable liquid lipid for development of NLCs containing ɤ-oryzanol.

In this study, ɤ-oryzanol incorporated NLCs with varying ratios of solid lipid to liquid lipid and total lipid concentration will be prepared with Tego® Care 165 (PEG-100 stearate mixed with glyceryl stearate, 50: 50 w/w) as solid lipid and cold pressed rice bran oil as liquid lipid. Furthermore, nonionic surfactant such as PEG-100 stearate Tween 80 and Tego® Care 450 will be used as a stabilizer in lipid system. This study focused on the NLCs development of these materials and characterized physicochemical properties of NLCs containing ɤ-oryzanol. The suitable formulation will be selected for stability testing in further study.

MATERIALS AND METHODS

Materials

Tego® care 165 (glyceryl stearate; PEG-100 stearate (50:50 w/w)) and Tego® care 450 (Polyglyceryl-3 methylglucose distearate) Tegin® M Pellets (Glyceryl stearate) were obtained from Goldschmidt (Germany). Tween 80 was purchased from KAO (Japan). ɤ-oryzanol was purchased from Wako Pure (Japan). Ultra-pure water was obtained from Mirae ST (Korea). Cold pressed Rice bran oil (CPRBO) from Thai Pathumthani fragrant rice (Oryza sativa L. var. indicav. Pathumthani 1, Thailand). Ethanol, methanol, isopropanol, dichloromethane, acetic acid and acetonitrile were obtained from Sigma-Aldrich (USA).
Methods

Preparation of cold pressed rice bran oil

Cold pressed Rice bran oil was prepared by screw press machine (Lopburi Vegetarian Oil Cold Pressed, Thailand) with two horse power and single phase induction motor and finally obtained crude oil. After that they were filtered through the white thin cloth, 10 and 2.5 µm filter paper respectively.

Preparation of nanostructured lipid carriers containing γ-oryzanol

NLCs containing γ-oryzanol were prepared by modified solvent injection technique. The oil phase composed of γ-oryzanol, solid lipid (Tego® Care 165 and/or Tegin® M Pellets), liquid lipid (Cold pressed rice bran oil and/or Caprylic/Capric triglyceride) and surfactant (Tego® Care 450 and/or Tween 80). They were heated and dissolved in ethanol until temperature more than melting point of solid lipid about 5°C and obtained lipid mixture. Ultrapure water containing Tween 80 was heated to approximate temperature of lipid mixture. After that, the aqueous phase was added to lipid mixture and obtained pre-emulsion. The pre-emulsion were homogenized by high speed homogenizer (IKA, Germany) at 13000 rpm for 2 minutes and then solvent was evaporated by rotary evaporator (BUCHI, Japan). The composition of NLCs showed in Table 1.

Table 1. Composition of nanostructured lipid carriers (%w/v)

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Ratio* (solid lipid: liquid lipid) w/w</th>
<th>Tego® Care 165**</th>
<th>Tegin® M Pellets</th>
<th>CPRBO</th>
<th>Caprylic/ Capric triglyceride</th>
<th>Tego® Care 450</th>
<th>Tween 80</th>
<th>γ-oryzanol</th>
<th>Water q.s. to</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-NLC1</td>
<td>9:2</td>
<td>4.5</td>
<td>0.5</td>
<td>-</td>
<td>2.5</td>
<td>3</td>
<td>0.2</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>γ-NLC2</td>
<td>8:4</td>
<td>4.0</td>
<td>1.0</td>
<td>-</td>
<td>2.5</td>
<td>3</td>
<td>0.2</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>γ-NLC3</td>
<td>7:6</td>
<td>3.5</td>
<td>1.5</td>
<td>-</td>
<td>2.5</td>
<td>3</td>
<td>0.2</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>γ-NLC4</td>
<td>9:2</td>
<td>9.0</td>
<td>1.0</td>
<td>-</td>
<td>2.5</td>
<td>3</td>
<td>0.2</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>γ-NLC5</td>
<td>8:4</td>
<td>8.0</td>
<td>2.0</td>
<td>-</td>
<td>2.5</td>
<td>3</td>
<td>0.2</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>γ-NLC6</td>
<td>7:6</td>
<td>7.0</td>
<td>3.0</td>
<td>-</td>
<td>2.5</td>
<td>3</td>
<td>0.2</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>γ-NLC7</td>
<td>9:2</td>
<td>4.5</td>
<td>0.5</td>
<td>-</td>
<td>2.5</td>
<td>-</td>
<td>0.2</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>γ-NLC8</td>
<td>9:2</td>
<td>4.5</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>0.2</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>γ-NLC9</td>
<td>9:2</td>
<td>4.5</td>
<td>0.5</td>
<td>-</td>
<td>3.5</td>
<td>4</td>
<td>0.2</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>γ-NLC10</td>
<td>9:2</td>
<td>4.5</td>
<td>0.5</td>
<td>0.5</td>
<td>2.5</td>
<td>3</td>
<td>0.2</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>γ-NLC11</td>
<td>9:2</td>
<td>-</td>
<td>4.5</td>
<td>0.5</td>
<td>2.5</td>
<td>3</td>
<td>0.2</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>γ-NLC12</td>
<td>9:2</td>
<td>4.5</td>
<td>0.5</td>
<td>-</td>
<td>2.5</td>
<td>3</td>
<td>0.4</td>
<td>100.0</td>
<td></td>
</tr>
</tbody>
</table>

* The ratio was expressed between solid lipid (Tego® care 165 or Tegin® M Pellets) and liquid lipid (CPRBO or Caprylic/ Capric triglyceride)
** Tego® care 165 is composed of glyceryl stearate and PEG-100 stearate (50:50, w/w) when calculation of the ratio (solid lipid: liquid lipid), the actual weight of solid lipid was calculated by only weight of glyceryl stearate.
Particle size and zeta potential

Particle size, size distribution and zeta potential of NLCs containing γ-oryzanol was measured by NanoPlus (Particulate Systems, USA) at 25 ± 0.2°C. The NLCs dispersion was diluted 50 folds with ultrapure water before measurement.

Entrapment efficiency and drug loading capacity

Entrapment efficiency of NLCs containing γ-oryzanol was determined by ultrafiltration centrifugation technique (Zhang et al., 2008). The centrifugal filter tubes (Amicon® Ultra0.5; Millipore, USA) with a molecular weight cut off of 30KDa were added NLCs 500 µl and centrifuged at 10,000 rpm for 10 minutes. Free drug which passed through membrane into collector tube was analyzed by High performance liquid chromatography (HPLC) on an Agilent 1260 infinity quaternary pump G1311B and Agilent 1260 infinity diode array detector G4212B equipped with Eclipse Zorbax XDB-C18 (250 mm × 4.6 mm, 5µm, Agilent, USA). The mobile phase composed of a mixture of methanol, acetonitrile, dichloromethane (55:35:9.5:0.5% (v/v)), flow rate was 1.4 ml/min at 25 ± 0.2°C (Ruktanonchai et al., 2009). γ-oryzanol was detected at 325 nm with a UV detector for 20 minutes. Entrapment efficiency and drug loading capacity of γ-oryzanol were calculated as follows.

\[
%\text{EE} = \left(\frac{\text{Drug}_T - \text{Drug}_F}{\text{Drug}_T}\right) \times 100
\]

\[
%\text{DL} = \left(\frac{\text{Drug}_T - \text{Drug}_F}{\text{Drug}_T - \text{Drug}_F + \text{C}_L}\right) \times 100
\]

Drugₜ is the amount of γ-oryzanol used in NLCs formulation
Drugₖ is the amount of γ-oryzanol in the water phase
Cₗ is the amount of lipid used to encapsulate drugs

Statistics

The data were expressed as mean ± SD. Significance of difference was evaluated by using Student’s T-Test at the probability level of 0.05.

RESULTS AND DISCUSSION

The physical appearance of NLCs containing γ-oryzanol was shown in Figure 1. The physical appearance of all formulations after the fresh preparation and one day storage were described in Table 2. After one day preparation at room temperature, they were found physical instability except for γ-NLC1 and γ-NLC9. γ-NLC1, γ-NLC2 and γ-NLC3 with various ratios of solid lipid to liquid lipid, they were found precipitation in γ-NLC2 and γ-NLC3. Corresponding with the study of Yang et al. (2014), it had been reported that amount of liquid lipid that affect to aggregation, which small amount of liquid lipid in NLCs was highly aggregated while the amount of liquid lipid increased that more gelation occurred. In addition, rice bran oil contained oleic acid about 38-40% (Cicero and Gaddi, 2001; Lin et al., 2009) and it was possible that oleic acid had an acid functional group, which might change interfacial properties of the oil-surfactant in an undesirable way (Yang et al., 2014). However, Tego® Care 165 was composed of glyceryl stearate and PEG-100 stearate (50: 50 w/w) and the ratio of γ-NLC1 was 9:2 that meant content of nonionic surfactant (PEG-100 stearate) in γ-NLC1 more than γ-NLC2 and γ-NLC3. It might be increasing the content of nonionic surfactants in γ-NLC1 stabilized the lipid system from precipitation. Thus, the suitable ratio of solid lipid: liquid lipid containing γ-oryzanol was 9: 2 (γ-NLC1). Increasing of total lipids to
two folds in γ-NLC4, γ-NLC5 and γ-NLC6; it caused precipitation because of excess lipid in the formulation. Thus, the appropriate content of total lipids was 2.75% (w/v). From these results, the ratio and the content of total lipids were chosen and developed for next formulations.

For γ-NLC7 and γ-NLC8, there were only two nonionic surfactants in the system that PEG-100 stearate with Tego® Care 450 and PEG-100 stearate with Tween 80, respectively. They could not stabilize the system. On the other hand, γ-NLC1 and γ-NLC9 had added three nonionic surfactants in the system which composed of Tego® Care 450, Tween 80 and PEG-100 stearate. They stabilized formulations when compared with the same ratio of solid lipid to liquid lipid. It was possibly when the increased content and type of nonionic surfactants that led to the steric hindrance to agglomeration between particles (Karn-orachai et al., 2014). This result contrasted with the result of Karn-orachai et al. (2014). It had been reported, mixed of two surfactants charges (nonionic and anionic or nonionic and cationic surfactant) showed less % crystallinity than three surfactant charges (nonionic, anionic and cationic surfactant) that indicated good stability better than. However, γ-NLC1 and γ-NLC9 still stabilized after one month preparation at room temperature. It was possible that steric hindrance of three nonionic surfactants promoted stability. In γ-NLC10, cold pressed rice bran oil was replaced by medium chain triglyceride that was caprylic/capric triglycerides and the separation was found. Moreover, γ-NLC11 was composed of only glyceryl stearate and absence of PEG-100 stearate. Gelation occurred in this formulation. This phenomenon involved in the physical instability such as large particle size and high polydispersity index value (PI) (Seetapan et al., 2010; Negi et al., 2014). Interestingly, gelation was found in γ-NLC12 when increased γ-oryzanol content. It was possible because parts of the γ-oryzanol structure were similar in structure of cholesterol, which it affected to instability in NLCs (Riangjanapatee et al., 2013). Thus, the concentration of γ-oryzanol was used at 0.2% w/v.

Table 2. Physical appearance of NLCs formulations after freshly prepare and 1 day storage

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Physical appearance</th>
<th>Physical appearance After 1 day</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-NLC1</td>
<td>Translucent colloid</td>
<td>Translucent colloid</td>
</tr>
<tr>
<td>γ-NLC2</td>
<td>Translucent colloid</td>
<td>Precipitation</td>
</tr>
<tr>
<td>γ-NLC3</td>
<td>Milky colloid</td>
<td>Precipitation</td>
</tr>
<tr>
<td>γ-NLC4</td>
<td>Milky colloid</td>
<td>Precipitation</td>
</tr>
<tr>
<td>γ-NLC5</td>
<td>Milky colloid</td>
<td>Precipitation</td>
</tr>
<tr>
<td>γ-NLC6</td>
<td>Milky colloid</td>
<td>Precipitation</td>
</tr>
<tr>
<td>γ-NLC7</td>
<td>Milky colloid</td>
<td>Separation</td>
</tr>
<tr>
<td>γ-NLC8</td>
<td>Milky colloid</td>
<td>Precipitation</td>
</tr>
<tr>
<td>γ-NLC9</td>
<td>Translucent colloid</td>
<td>Translucent colloid</td>
</tr>
<tr>
<td>γ-NLC10</td>
<td>Translucent colloid</td>
<td>Separation</td>
</tr>
<tr>
<td>γ-NLC11</td>
<td>Milky colloid</td>
<td>Gelation</td>
</tr>
<tr>
<td>γ-NLC12</td>
<td>Milky colloid</td>
<td>Gelation</td>
</tr>
</tbody>
</table>
Physicochemical properties of γ-NLC1 and γ-NLC9 were shown in Table 3. The particle sizes of both formulations were in the range 100-200 nm. The particle size of γ-NLC9 was a significant difference when compared with the particle size of γ-NLC1 (p<0.05) because of the high concentration of surfactant in γ-NLC9. It has been reported by Palumbo et al. (2002) that the increasing amount of nonionic surfactant might cause a reduction of the polymeric wall thickness of the particle which reduced particle size. Moreover, blank NLC9 showed a significant difference of the particle size when compared with γ-NLC9 (p<0.05) that referred to γ-oryzanol had an influence on the particle size of NLCs. PI values of both formulations were less than 0.15, indicating narrow size distribution of particle.

Zeta potential values showed a significant difference (p<0.05) between γ-NLC1 and γ-NLC9. γ-NLC9 had zeta potential values about ± 30 mV that indicated good physical stability of the colloidal system (Souto et al., 2005; Duman and Tunc, 2009). Interestingly, the presence of three nonionic surfactants in system stabilized formulations better than two nonionic surfactants in the system. It was possible that PEG-100 stearate, Tween 80 and Tego® Care 450 caused steric hindrance which improved the stability of colloidal system (Lim and Kim, 2002; Ruktanonchai et al., 2009; Kyadarkunte et al., 2015). The pH values of both formulations were nearly neutral pH (about 7).

The entrapment efficiency of γ-NLC1 and γ-NLC9 could encapsulate more than 99% consistent with the results were observed by polarized light microscopy, which no drug crystal found in NLCs (data not shown). This result was due to the lipophilicity of γ-oryzanol. It could highly solubilize in cold pressed rice bran oil which led to a high packing ability of γ-oryzanol in the lipid matrix of NLCs and caused reduced crystallinity and increase imperfections in the crystal lattice (Rahman et al., 2013). In similar to the previous reports;
lipophilic drug could encapsulate approximately 100% in NLCs by using glyceryl stearate as solid lipid (Teeranachaidikul et al., 2008). Drug loading capacity of γ-NLC1 and γ-NLC9 showed 6.75 ± 0.01% and 6.73 ± 0.02% respectively. They had approximate values because both formulations had the same ratio of total lipids.

**Table 3.** Physicochemical characterization of NLCs containing γ-oryzanol

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Z-ave (nm)</th>
<th>PI</th>
<th>Zeta potential (mV)</th>
<th>pH</th>
<th>Entrapment Efficiency (%)</th>
<th>Drug loading capacity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-NLC1</td>
<td>165.4 ± 6.0*</td>
<td>0.121 ± 0.014</td>
<td>-20.44 ± 1.97*</td>
<td>6.78 ± 0.03</td>
<td>99.62 ± 0.11</td>
<td>6.75 ± 0.01</td>
</tr>
<tr>
<td>γ-NLC9</td>
<td>133.9 ± 0.9</td>
<td>0.132 ± 0.017</td>
<td>-30.51 ± 2.31</td>
<td>7.12 ± 0.01</td>
<td>99.78 ± 0.01</td>
<td>6.73 ± 0.02</td>
</tr>
<tr>
<td>Blank-NLC9</td>
<td>108.53 ± 1.02*</td>
<td>0.137± 0.008</td>
<td>-26.78 ± 1.92</td>
<td>7.11 ± 0.02</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Mean ± S.D. (n = 3)

* = significant difference when compared with γ-NLC9 (p<0.05)

**CONCLUSION**

The suitable formulation of γ-oryzanol-loaded NLCs is γ-NLC9. They base on the mixture of triglycerides blended PEG-100 stearate (solid lipid) with cold pressed rice bran oil (liquid lipid) and their ratio is 9: 2 (solid lipid: liquid lipid) and the content of total lipids is 2.75% (w/v). Both compositions are appropriate for development of γ-oryzanol-loaded NLCs. They increase γ-oryzanol encapsulation approximate 100%. Interestingly, three combinations of nonionic surfactant between PEG-100 stearate, Tween 80 and Tego® Care 450 not only decrease particle sizes but also stabilize better than two combinations of nonionic surfactant. For further study, the suitable formulation will be prepared by different methods and investigated physicochemical properties and studied formulation stability.

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