Effect of liquid crystals with cyclodextrin on the bioavailability of a poorly water-soluble compound, diosgenin, after its oral administration to rats.

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Abstract

Diosgenin, found in wild yam (*Dioscorea villosa*), has been shown to ameliorate diabetes and hyperlipidemia, increase cell proliferation in a human 3D skin model, and inhibit melanin production in B16 melanoma cells. It is also an active element in cosmeceutical and dietary supplements. Although the bioavailability of diosgenin is low due to its poor solubility and intestinal permeability, it was subsequently improved using a β-cyclodextrin (β-CD) inclusion complex. Recently liquid crystals (LCs) were shown to enhance the bioavailability of poorly water-soluble drugs. The purpose in the present study was to prepare diosgenin LCs and investigate the interaction between LC and β-CD in order to improve its bioavailability of diosgenin. Crystallinity and particle diameters of LCs in water were determined by small angle X-ray scattering (SAXS) and Zetasizer. Pharmacokinetic parameters were calculated using the plasma content of diosgenin after its oral administration to Wistar rats. Regarding the formation of glyceryl monooleate (GMO) and phytantriol (PHY) LC, SAXS patterns showed the hexagonal and cubic phases, respectively. Bioavailability was significantly enhanced after oral administration of LCs prepared by GMO than after diosgenin alone. The bioavailability was father improved with the combination of LC and β-CD than LC and water.

Keywords: Diosgenin, liquid crystal, glyceryl monooleate, phytantriol, β-cyclodextrin, oral administration.

1. Introduction

Many chemical compounds have recently been synthesized and characterized to develop new drug candidates in pharmaceutical industries. However, more than 40% of these compounds have been terminated due to poor dissolution and/or biomembrane permeability (Prentis et al., 1988; Venkatesh and Lipper, 2000). Poorly water-soluble compounds have been detected not only in medicines, but also in dietary and cosmetic supplements. Nevertheless, few studies have investigated the pharmacokinetics of these supplements. Thus, the pharmacokinetics of these supplements need to be investigated to improve their oral absorption. Diosgenin, which is extracted from wild yam (*Dioscorea villosa*) and fenugreek (*Trigonella foenum greaceum*), is a steroidal saponin (Taylor et al., 2000; Hooker, 2004). In our previous study, we demonstrated that the solubility of diosgenin in water and its bioavailability were poor (Okawara et al., 2010). The oral administration of diosgenin to diabetic rats significantly decreased plasma glucose levels (Pari et al., 2012). Furthermore, diosgenin decreased serum total cholesterol, triglyceride, and low-density lipoprotein cholesterol levels in rats fed a high-fat diet (Gong et al., 2010). Diosgenin has been established as a raw material for the production of steroidal hormones in the pharmaceutical industry (Applezweig, 1969). It has also been used as dietary supplement in hormone replacement therapy for menopausal women (Russell et al., 2002; Benghuzzi et al., 2003). Orally administrated diosgenin was shown to improve skin thickness in ovariectomized mice, and enhanced DNA synthesis in a human 3D equivalent model (Tada et al., 2009). Furthermore, it inhibited melanogenesis in B16 melanoma cells by activating the phosphatidylinositol-3-kinase pathway (Lee et al., 2007). Based on these findings, diosgenin is considered as an active element in cosmeceutical and dietary supplements. We previously reported that the bioavailability of diosgenin was only 6% in rats. We have been

66 investigating ways by which to improve its low bioavailability. Our findings suggested that
67 diosgenin and β-cyclodextrin (β-CD) formed 1:2 molar ratio inclusion complexes that
68 improved the bioavailability of diosgenin to 45% in rats (Okawara et al., 2013). However,
69 the β-CD inclusion complex has to be suspended in water when administered to rats, and it
70 also takes time to prepare the complex.
71 Liquid crystals (LCs) are semisolids with crystalline structures combining the properties
72 of both solid and liquid states (Yamada et al., 2011). Commonly encountered phases in LCs
73 include the lamellar, bicontinuous cubic, and inverse hexagonal phases (Clogston et al.,2000).
74 LCs are easily formed by various amphipathic lipids such as glyceryl monooleate (GMO) and
75 phytantriol (PHY) in excess amounts of water (Lee et al., 2009; Costa-Balogh et al., 2010).
76 Our previous report must be the first one for the LC using PHY (Yamada et al., 2011). Many
77 studies reported that the oral administration of LC enhanced the bioavailability of poorly
78 water-soluble drugs (Boyd et al., 2007; Nguyen et al., 2011; Lian et al., 2011). In this study,
79 we prepare self-assembly LCs and dispersed LCs including diosgenin, and physicochemical
80 measurements for LCs were performed using Zetasizer and small angle X-ray scattering
81 (SAXS). LCs were administered to rats and their pharmacokinetic parameters were calculated
82 for diosgenin. Furthermore, we elucidated the interaction between LC formation and β-CD
83 solution for oral administration to rats.
84
85 **2. Materials and methods**
86 **2.1. Materials**
87 Diosgenin was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Polyoxyethylene
88 hydrogenated castor oil 60 (HCO-60) was supplied from Nikko Chemicals (Tokyo, Japan).
89 Sodium pentobarbital was obtained from Kyoritsu Seiyaku (Tokyo, Japan). GMO and PHY

were obtained from Tokyo Chemical Industry (Tokyo, Japan). β-CD, 6-methyl diosgenin, and other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Preparation of liquid crystals

LC was formed using GMO or PHY and an equal or greater volume of water, and involved a transition from the lamella phase to the hexagonal phase, and finally to the cubic phase by heating (Lee et al., 2009). To determine the solubility of diosgenin in lipids, each lipid was added to a tube with an excess amount of diosgenin. The mixture was heated at 37°C in a heating block to facilitate solubilization using a vortex mixer. Mixtures were shaken in a thermo-controlled incubator at 37°C for 2 h. After reaching equilibrium, each tube was centrifuged at 15,000 ×g for 5 min and the supernatant was collected. After dilution, the content of diosgenin was determined using a liquid chromatography mass spectrometry (LCMS) system. Separation was achieved by an MXY01-01 (Michrom Biosources Inc., CA, U.S.A.) with a TSK gel ODS-100V column (2.0×50 mm, 3 μm) (TOSOH, Tokyo, Japan) at room temperature. The mobile phase consisted of methanol (90%) and H_2O (10%) containing 10 mM ammonium acetate. The flow rate was set to 150 μL/min and detection was performed using an LCQ DECA XP ^{Plus} mass spectrometer (Thermo Fisher Scientific Inc., MA, U.S.A.). Self-assembly LCs were prepared by dissolving diosgenin in each lipid at 5 mg/mL by heating at 70°C.

2.3. SAXS measurement

Equal volumes of water and self-assembly LC were mixed prior to measurements being taken. These samples were heated at 70°C, and mixed to homogenization using a vortex mixer. SAXS measurements were performed on a NANO-Viewer (Rigaku) with PILATUS 100K/RL

114 2D detector. The X-ray source was Cu Kα radiation, wavelength 1.54 Å, operating at 45 kV
115 and 110 mA. The sample-to-detector distance was chosen to be 375 mm. Each sample was
116 placed between polyether ether ketone membranes and exposed for 10 min.
117
118 **2.4. Dispersibility of LC**
119 Dispersed LC was prepared by adding 10-fold of water to self-assembly LC. The
120 mixture was heated on a heating block at 70°C, and shaken using a vortex mixer. These
121 samples were centrifuged at 15,000 ×g for 5 min, and the water phase was collected. Samples
122 were diluted 1000-fold in water prior to measurements being taken. Particle diameters and
123 dispersion were measured by dynamic light scattering measurements on a Nano-ZS ZEN 3600
124 Zetasizer (Malvern, Worcestershire, UK) with water.
125
126 **2.5. Solubility study**
127 In the solubility study, 10-fold of water or 4 mM β-CD solution were added to
128 self-assembly LC, and mixed at 37°C for 5 days. These samples were centrifuged at 15,000×g
129 for 5 min, and the water phases were collected. The contents of diosgenin and each lipid were
130 determined using an LCMS system after filtration with a 0.2 μm membrane filter (ADVANTEC,
131 Tokyo, Japan) and dilution.
132
133 **2.6. Animals**
134 Male Wistar rats (200 to 250 g) were provided from Japan SLC (Hamamatsu, Shizuoka,
135 Japan). Animals were housed under a 12 h light and dark cycle in a temperature-controlled
136 room (23 ± 2°C). They had free access to food and water. The animal care protocol was
137 approved by the Animal Care and Use Committee of Josai University (Saitama, Japan).

138

139 **2.7. Pharmacokinetic studies**

140 Intravenous and oral administration studies were performed to compare the
141 pharmacokinetic parameters of diosgenin and its LC. Rats were fasted from at least 12 h prior
142 dosing to 4 h after dosing. In the intravenous administration protocol, diosgenin was dissolved
143 in saline containing 1% HCO-60, and 121 μg/kg (body weight) of diosgenin was injected into
144 the tail vein. For oral administration, a diosgenin suspension of 5 mg/mL or self-assembly LC
145 formulation containing an equivalent amount of diosgenin was prepared and administered at a
146 dose of 2 mL/kg (body weight). Diosgenin was suspended in saline containing 1% HCO-60.
147 Self-assembly LC was prepared by dissolving diosgenin in PHY or GMO. In the LC group,
148 self-assembly LC and equal amounts of water or β-CD solution (24 mM) were simultaneously
149 administrated. Blood was collected from the tail vein into heparinized tubes at times ranging
150 from 0 to 96 h, and was immediately separated by centrifugation. In a previous study, skin
151 levels of diosgenin peaked 6 h after its oral administration (Okawara et al., 2013). Skin
152 samples were taken from the entire abdomen 6 h after the oral administration. Each sample
153 was stored at -30°C until analyzed.

154

155 **2.8. Analytical procedure**

156 6-methyl diosgenin was used as an internal standard to assess diosgenin levels in plasma
157 and skin samples. Samples were added to methanol and extraction was achieved by sonication
158 for 20 min at 37°C. These samples were centrifuged at 15,000 × g for 5 min and the
159 supernatants were collected. The content of diosgenin was determined by an LCMS system
160 with a Tosoh TSK gel ODS-100V column (2.0×50 mm, 3 μm) at room temperature.

161

2.9. Pharmacokinetics and statistical analysis

Pharmacokinetics analysis was performed with nonlinear least-squares fitting. The area under the plasma concentration-time curve (AUC) was calculated using the linear trapezoidal rule. The absolute bioavailability was determined as AUCpo/AUCiv, using mean AUC values for oral and intravenous doses. Tukey's multiple comparison tests was used to assess the significance of differences between groups. A p value of less than 0.05 was considered significant.

3. Results

3.1. SAXS measurements

The phase behavior of LCs made from GMO or PHY was confirmed by SAXS measurements. Representatives of the SAXS profiles of LCs are shown in Figure 1. SAXS curves revealed the presence of a hexagonal phase (with reflections spaced at √1, √3, and √4; Fig. 1A) for GMO LC and bicontinuous cubic phase (reflections at √2, √3, √4, √6, √8, √9, √10, and √12; Fig. 1B) for PHY LC. The phase behavior of LCs was not affected by the presence of diosgenin (see the lines a and b). These results confirmed that these lipids formed LCs when mixed with water at room temperature.

3.2 Dispersibility of LC

The particle diameter and dispersion of LCs were measured to confirm their conditions in the gastric and intestinal tract. The particle diameters of LCs made from GMO and PHY are listed in Table 1. Each particle diameter of LCs was nearly 100-200 nm and its distribution in water remained unchanged in the presence of diosgenin. PHY LC showed larger diameter than GMO LC both with and without diosgenin. Although the diameter of a particle of PHY became small by diosgenin, it is unknown for details.

3.3. Solubility study

The solubility of diosgenin and lipids in water or β-CD solution are listed in Table 2. The solubility of diosgenin was increased by β-CD and GMO, whereas it was decreased by PHY. It is suggested that the lower release of diosgenin was caused by PHY melting point (70°C). More diosgenin was released from LCs in β-CD solution than in water. Correspondingly, the solubilities of GMO and PHY in water were increased by β-CD.

194

195 **3.4. Pharmacokinetics of diosgenin self-assembly LC with CD solution after oral**

196 **administration**

197 Fig. 2 shows the mean plasma diosgenin concentration-time curves after oral

198 administration of self-assembly LC and CD solution in Wistar rats. Table 3 shows the

199 calculated pharmacokinetics parameters. The maximum plasma concentration of diosgenin

200 (Cmax) after oral administration of GMO LC was higher than of the diosgenin suspension.

201 The time to reach the maximum plasma concentration (Tmax) of GMO LC was similar to that

202 of the suspension (Fig. 2A). However, the Tmax was significantly higher and Cmax was

203 significantly lower with PHY LC than with the diosgenin suspension (Fig. 2B). Plasma

204 concentrations were higher in the β-CD combination groups than in self-assembly LC with

205 water. Diosgenin bioavailability was significantly higher after the oral administration of GMO

206 LC with β-CD solution than after GMO LC with water.

207

208 **3.5. Skin distribution of diosgenin**

209 The distributions of diosgenin in skin were determined after the oral administration of

210 diosgenin or its LC and summarized in Figure 3. The contents of diosgenin in skin 6 h after its

211 administration alone, with β-CD solution, GMO LC, GMO LC with β-CD solution, PHY LC,

212 and PHY LC with β-CD solution were 23.95, 90.70, 178.21, 371.79, 11.39, and 8.93 ng/g skin,

213 respectively. The content of diosgenin was significantly higher in the GMO LC with β-CD

214 solution groups than in the diosgenin suspension.

4. Discussion

We initially prepared diosgenin LCs and characterized their physical properties. Self-assembly LCs and dispersed LCs were prepared and analyzed using SAXS and Zetasizer. The SAXS data demonstrated that GMO and PHY, which dissolved diosgenin, formed hexagonal and cubic phases, respectively, when mixed with equal volumes of water at room temperature. The particle diameter of LCs was nearly 100-200 nm in water. These results suggested that GMO and PHY LCs are dispersed in the gastrointestinal tract as microdroplets (Jin et al., 2013). The solubility of diosgenin was increased by β-CD and GMO. Further enhancements in its solubility were obtained when LCs were combined with β-CD solution. These results indicated that β-CD solution enhanced the release of diosgenin and lipids from LCs.

Plasma diosgenin levels were relatively low after oral administration of the diosgenin suspension, and were similar to those in our previous study (Okawara et al., 2013). However, GMO LC significantly improved its bioavailability. Previous studies reported that GMO LC were strongly bioadhesive and stimulated entrapped-drug permeation through the intestinal mucosa (Geraghty et al., 1997; Lai et al., 2009). Tmax was higher with PHY LC than with the diosgenin suspension. Nguyen et al. suggested that PHY LC retained in the gastrointestinal tract may slowly diffuses, while suppressing drug release; this is consistent with the results of the present study (Nguyen et al., 2010). Furthermore, the combination of self-assemble LC and β-CD solution enhanced the plasma concentration and bioavailability more than LC alone. Different patterns were confirmed in plasma diosgenin concentration–time curves after the oral administration of GMO LC and PHY LC. These may have been caused by differences in the melting points of lipids than changes in LC phases. The skin distribution of diosgenin was higher after the oral administration of GMO LC and GMO LC with β-CD solution than that of

239 the diosgenin suspension. A lower skin content was observed after the oral administration of
240 PHY LC and PHY LC with β-CD solution. A previous study reported a correlation between
241 plasma concentrations of diosgenin and skin content (Okawara et al., 2013). These findings
242 suggested that PHY LC and PHY LC with β-CD solution maintained the skin content of
243 diosgenin over 48 h. Although further research is needed, self-assembly LCs may control the
244 release of drugs included in LC. Furthermore, the results of the present study indicate that
245 β-CD solution improve the release of drugs from LCs.
246
247 **Conclusions**
248 The aim of this study was to improve the bioavailability of diosgenin. We prepared
249 diosgenin LCs and evaluated their combined effect with β-CD solution. The solubility,
250 bioavailability, and skin distribution of diosgenin were much better with LC in β-CD solution
251 than that with LC alone. These results indicate that β-CD solution increases diosgenin and
252 lipid release from LCs.

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315 **Figure legends**

316 Figure 1. SAXS profiles for GMO (A) and PHY (B). The profiles are shown for LC with
317 diosgenin (a) and without diosgenin (b). LCs were prepared by mixing equal volumes of
318 self-assembly LC and water at 70°C.

319

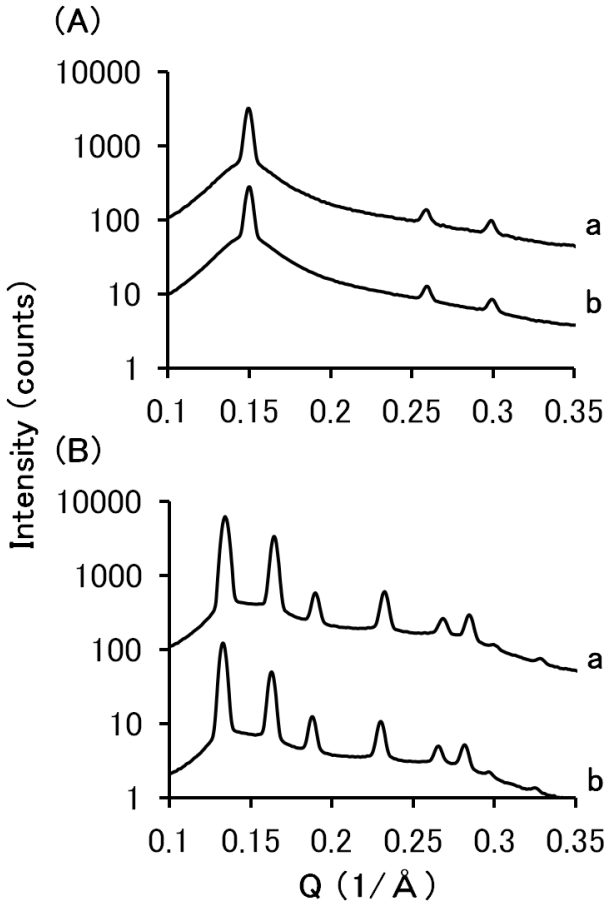
320 Figure 2. Plasma profiles after the oral administration of diosgenin: A- (○), diosgenin
321 suspension; (□), GMO self-assembly LC with water; (■), GMO self-assembly LC with β-CD
322 solution; B-(○), diosgenin suspension; (□), PHY self-assembly LC with water; and (▲), PHY
323 self-assembly LC with β-CD solution. Each point shows the mean ± S.E. of 3 to 8
324 experiments. *: $p<0.05$ significantly different from the diosgenin suspension. †: $P<0.05$
325 significantly different from its self-assembly LC (Tukey's test).

326

327 Figure 3. Skin distribution of diosgenin after oral doses of diosgenin and LC. Skin samples
328 were collected 6h after the oral administration of diosgenin and LC. Each column shows the
329 mean ± S.E. of 3 to 4 experiments. *: $p<0.05$ significantly different from the diosgenin
330 suspension (Tukey's test).

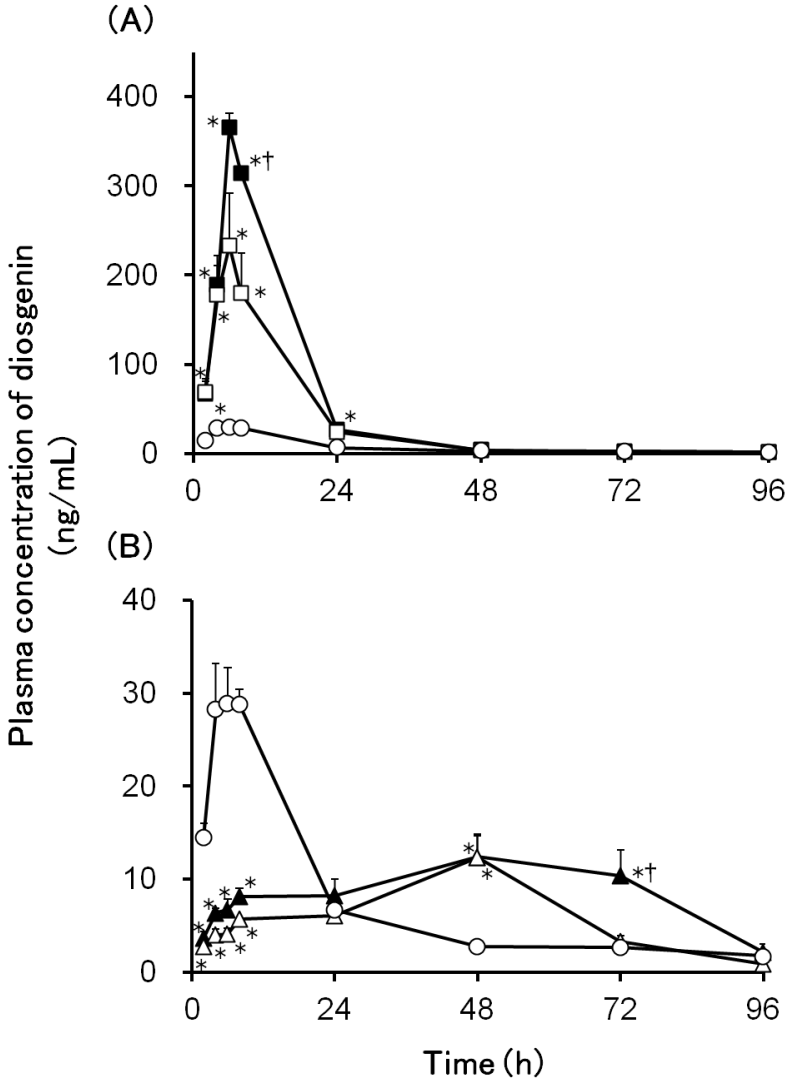
331 **Figures**

332 Fig. 1



333

334 Fig. 2


(A)
400
300
200
100
0
0
24
48
72
96
(B)
40
30
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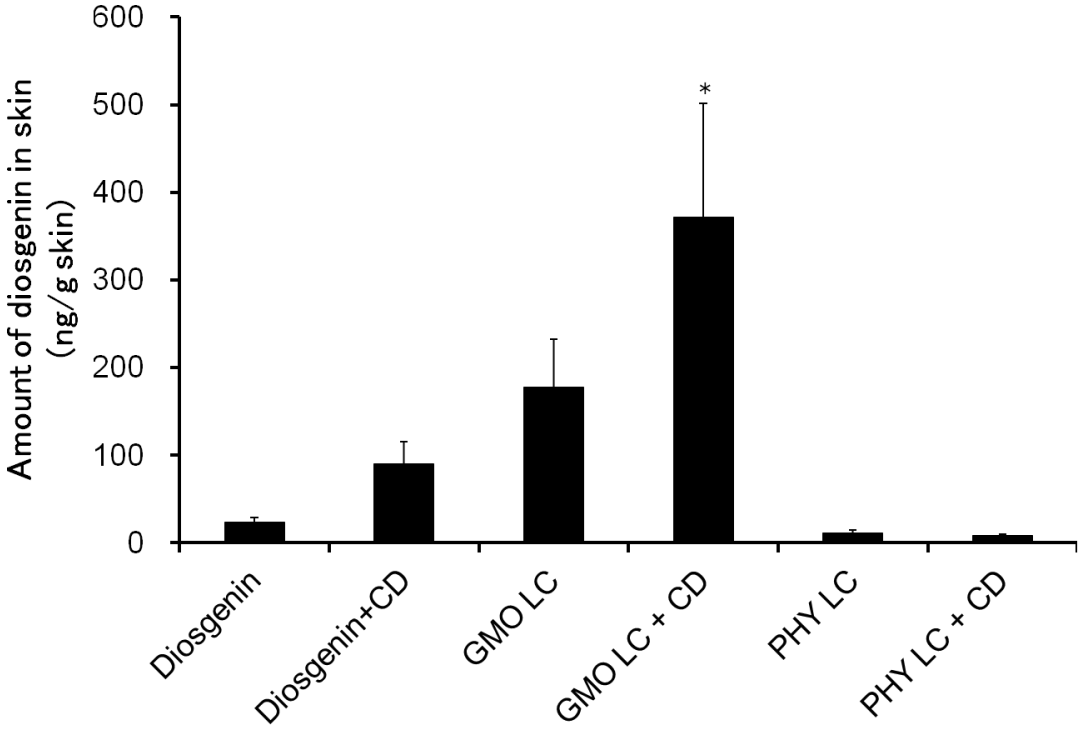
10
0
0
24
48
72
96
Plasma concentration of diosgenin (ng/mL)
Time (h)

335

336 Fig. 3



337

338

339 **Tables**

340 Table 1. Particle diameter of LCs.

	Particle diameter (nm)	
	Without diosgenin	With diosgenin
GMO	112.1 ± 0.5	117.6 ± 0.5
PHY	212.1 ± 0.8	138.1 ± 0.5

341 Self-assembly LCs were dispersed in water at 37°C. The particle diameter of each formation

342 was measured after centrifugation and dilution.

343

344

345

346 Table 2. Solubility of diosgenin and lipids in water or β-CD solution.

	Solubility of diosgenin in water（ng/mL）		**CD/Water ratio**	**Solubility of lipids in water（μg/mL）**		**CD/Water ratio**
	Water	**CD solution**		**Water**	**CD solution**	
GMO	2,210 ± 460	8,360 ± 400	3.77	409 ± 40	464 ± 112	1.14
PHY	4.50 ± 1.11	3,550 ± 220	789	3.99 ± 0.48	16.9 ± 1.9	4.24
Without lipids	10.5 ± 3.5	3,260 ± 170	310	-	-	-

347 Diosgenin was dissolved in GMO or PHY at 5 mg/mL. A ten-fold amount of water or 4 mM

348 β-CD solution was added, and mixed at 37°C for 5 days. Each value shows the mean ± S.E. of

349 3 experiments.

350

351

352 Table 3. Pharmacokinetic parameters after the oral administration of the diosgenin suspension
353 and self-assembly LCs with water or CD solution.

	AUCpo (ng・h/mL)	*Cmax* (ng/mL)	*Tmax* (h)	Bioavailability (%)	Enhancement ratio
GMO LC + CD	4403 ± 132*†	239 ± 2*	5.6 ± 0.2	47.1 ± 1.4*†	6.2
GMO LC	2934 ± 465*	164 ± 36*	6.0 ± 1.2	31.4 ± 5.0*	4.1
PHY LC + CD	1000 ± 191	11.4 ± 1.7*	22 ± 3*	10.2 ± 1.8	1.4
PHY LC	633.9 ± 62.0	8.38 ± 0.52*	22 ± 4*	7.30 ± 0.05	0.9
Diosgenin	711.4 ± 53.3	23.0 ± 1.9	5.0 ± 0.7	7.61 ± 0.51	1.0

354 Each value shows the mean ± S.E. of 3 to 4 experiments. *: $p<0.05$ significantly different
355 from diosgenin. †: $p<0.05$ significantly different from its LC. (Tukey's test).
356