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International Journal of Pharmaceutics 124 (1995) 19–26

international journal of pharmaceutics

Effects of soybean-derived sterol and its glucoside mixtures added in dipalmitoylphosphatidylcholine liposomes on the blood circulation and hepatic cellular distribution in mice

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Received 25 November 1994; revised 30 January 1995; accepted 18 February 1995

Abstract

The effects of a soybean-derived sterol mixture (SS) and their glucoside mixture (SG) in dipalmitoylphosphatidylcholine (DPPC) liposomes on blood circulation and hepatic distribution were investigated by measuring the leakage of calcein after intravenous administration to mice. Four kinds of liposomes were prepared: liposomes consisting only of DPPC (DPPC liposomes); and DPPC liposomes containing SS, cholesterol (Ch) or SG (molar ratio of DPPC/X = 7:2, X = SS, Ch, SG; SS, Ch and SG liposome), respectively. The area under the blood concentration-time curve (AUC) was greater in the order, SS liposomes > DPPC liposomes > SG liposomes, and the order is the same for lower membrane fluidity. Hepatic cellular distribution of SG liposomes 2 h after intravenous injection was significantly high compared with that of DPPC and SS liposomes. The results indicated that SS liposomes were stable in the blood circulation; however, SG liposomes were not stable and appeared to have an enhancing effect on hepatic uptake. This difference might indicate that hepatic accumulation is primarily governed by the glucose group of SG.

Keywords: Liposome; Dipalmitoylphosphatidylcholine; Soybean-derived sterol; Sterylglucoside; Stability; Blood circulation; Hepatic cellular distribution

1. Introduction

Liposomes have been investigated for their potential use as drug carriers for many years (Senior, 1987). Systemically administered liposomes are rapidly taken up by the reticuloendothelial system (RES), primarily Kupffer cells and splenic macrophages (Hwang, 1987). Mannose and galactose have often been used in the targeting of liposomes and polymers to Kupffer cells and parenchymal cells, respectively (Leserman and Machy, 1987). Recently, lactose carrying polystyrene has been reported to be effective in targeting to parenchymal cells (Goto et al., 1994). The lactose group is a liver-specific targeting material. However, the glucose group is not known to favor a specific biodistribution.

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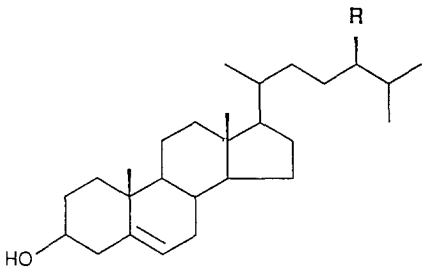
SSDI 0378-5173(95)00068-2



R= CH_3 : campesterol
R= C_2H_5 : sitosterol
R= C_2H_5 and Δ^{22} : stigmasterol
R= CH_3 and Δ^{22} : brassicasterol

Fig. 1. Chemical structures of soybean-derived sterols (SS).

We have reported that dipalmitoylphosphatidylcholine liposomes (DPPC liposomes) with a soybean-derived sterol mixture (SS) and its glucoside mixture (SG) are very different in physicochemical properties in vitro (Muramatsu et al., 1994). The SG is a mixture of monoglucosides of β-sitosterol (49.9%), campesterol (29.1%), stigmasterol (13.8%), and brassicasterol (7.2%) as shown in Fig. 1. SS is obtained by the hydrolysis of the glucoside bond of SG, i.e., SS is the aglycon of SG. SS stabilized the DPPC liposomes to a greater extent than cholesterol (Ch), which is usually used as a stabilizer, in a molar ratio of DPPC/X = 7:2, X = Ch, SS, but SG appears to fluidize the membrane (Muramatsu et al., 1994; Qi et al., 1995). These differences in the in vitro behavior of SS and SG may be due to the glucose group of SG; therefore, it is of interest to examine the in vivo behavior.

In this study, the effects of DPPC liposomes with SS or SG entrapping calcein on stability in blood and hepatic cellular distribution were investigated by measuring the release of calcein compared with an in vitro experiment incubating liposomes in plasma.

2. Materials and methods

2.1. Materials

DPPC and Ch were purchased from Sigma Chemical Co. (St. Louis, MO). Calcein was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). SS and SG were kindly supplied by Ryukakusan Co., Ltd (Tokyo, Japan). Sodium pentobarbital (Nembutal®, Dainabot, Osaka, Japan), Hank's balanced salt solution (Life Technologies, Inc., NY) and collagenase (Wako Pure Chemical Industries, Ltd, Osaka, Japan) were purchased commercially. All other chemicals used were of reagent grade.

Male ddY mice weighing 27–32 g, purchased from Saitama Experimental Animal Supply (Saitama, Japan), were used in all experiments. For plasma incubation, the plasma of Wistar rats was used.

2.2. Preparation of liposomes

Multilamellar liposomes were prepared according to a standard method (Bangham et al., 1974) as described in a previous study (Muramatsu et al., 1994). Briefly, the appropriate lipids were dissolved in chloroform and dried under reduced pressure. The resultant lipid film (70 μmol DPPC) was then hydrated in 3 ml of pH 7.4 phosphate-buffered saline (PBS) containing calcein as an aqueous marker. The mixture was then vortexed, followed by sonication in a bath-type sonicator (Honda Electronics, W220R, Tokyo, Japan) and centrifugation at $9500 \times g$ for 5 min to remove large particles and form a homogeneous size. Non-entrapped calcein was removed by gel filtration. DPPC liposomes with Ch, SS or SG (Ch, SS or SG liposomes) were composed of DPPC/Ch, SS or SG in a molar ratio of 7:2, respectively. The size of Ch, SS and SG liposomes was determined to be 83.6–96.9, 139.6–157.1 and 100–117 nm, respectively, using a Sub-Micron Particle Analyzer (Coulter model N4, Coulter Co.).

2.3. Fluorescence anisotropy measurement

The precise method of fluorescence anisotropy measurement was previously reported (Muramatsu et al., 1994). The fluorescence anisotropy with diphenylhexatriene (DPH) in liposomes was measured at 37° C.

2.4. Leakage of calcein from liposomes in plasma in vitro

Each liposome suspension (0.5 ml, 0.95 mg of total lipids) was added to rat plasma (0, 25, 50, 90% (v/v)) and incubated at 37 ± 0.5° C. 0, 10, 20, 30, 40, 50 and 60 min after addition of the liposomes, the percentage of calcein released from liposomes was calculated from the fluorescence intensities (excitation at 490 nm, emission at 520 nm) with and without Triton X-100 treatment according to the following equation;

$$\%\ \text{of calcein release} = 100(F_t - F_R)/(F_\infty - F_R) \quad (1)$$

where F_R is the fluorescence intensity of the suspension of liposomes entrapping calcein, F_∞ denotes the fluorescence intensity of calcein when the liposomes entrapping calcein were completely disrupted by Triton X-100 and F_t is the fluorescence intensity of calcein at time t.

2.5. Blood circulation and biodistribution

Mice were intravenously injected via the tail vein with a dose of liposome suspension in PBS (6.7 ml/kg, 0.38 mg of total lipids). 5, 10, 15, 20, 30, 60, 120, 240 and 360 min after the injection, a 10 μl sample of blood was drawn from the tail vein ($n = 3$). The samples were immediately poured into 2.4 ml of PBS and well mixed, then centrifuged at 1000 × g for 15 min, and 1 ml of the supernatant solution without hemolysis was added to 100 μl of 0.275 M EDTA according to the method of Sawahara et al. (1991).

In addition, 360 min after injection, the mice were killed by cervical dislocation, and the liver and spleen were removed. The whole liver and spleen were homogenized with 25 ml of PBS and then centrifuged at 1000 × g for 30 min, and 1 ml of filtered supernatant solution was added to 100 μl of 0.275 M EDTA. In these experiments, the fluorescence intensity of calcein from liposomes was calculated according to Eq. 1. The area under the blood concentration-time curve (AUC) was calculated based on the trapezoidal rule from 0 to 6 h.

2.6. Fractionation of liver cells

The liposome suspension (6.7 ml/kg) was intravenously injected via the tail vein of mice which were anesthetized by an i.p. injection of sodium pentobarbital (2 ml/kg body wt) and 200 IU of heparin was injected intraperitoneally.

Liver cells were dispersed from mouse liver according to the method of Shimaoka et al. (1987). The liver was perfused in situ through the portal vein with Ca-free Hank's balanced salt solution containing 0.5 mM EGTA at 37° C for 5 min, and washed with PBS for 5 min and then perfused with 0.05% collagenase solution for 10 min. The perfused liver was minced, dispersed in Hank's solution, and filtered. The liver cells were separated into subfractions according to the method of Yamashita et al. (1991). Purified parenchymal

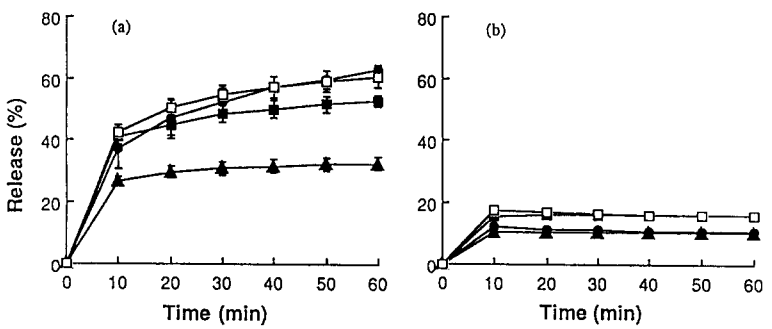


Fig. 2. Release profiles of calcein from liposomes incubated with 50% rat plasma (a) or PBS (b) at 37 ± 0.5° C (DPPC/X = 7:2; X = SS, Ch, SG). Each value represents the mean ± S.D. of three mice. DPPC liposomes (●), SS liposomes (▲), Ch liposomes (■), SG liposomes (□).

cells were obtained by centrifugation at $50 \times g$ for 1 min. The precipitated cells were washed twice with Hank's solution. The supernatant obtained via the first centrifugation and the washed solution were combined and centrifuged at $70 \times g$ for 1 min. Similarly, a third centrifugation was carried out at $140 \times g$ for 1 min. The viabilities of parenchymal and non-parenchymal cells were examined by the trypan-blue exclusion test and were more than 70%. Parenchymal cell numbers were counted in a Burker-Turk chamber.

2.7. Statistical analysis

Data from the animal experiments using mice were compared using analysis of variance and Student's *t*-test. A p value of 0.05 was considered significant.

3. Results

3.1. Stability of liposomes in plasma in vitro

The time courses of the release (%) of calcein entrapped in DPPC, SS, Ch and SG liposomes during incubation with 50% rat plasma and PBS at 37.5° C are shown in Fig. 2a and b, respectively. The release of the marker from each liposome in PBS was less than 20% up to 60 min, but about 40% of the marker was released within 20 min when exposed to rat plasma, except for SS liposomes. The release of calcein into plasma is much greater than that into PBS from the liposomes. The release of calcein in 50% rat plasma was greater in the following order: DPPC liposomes = SG liposomes ≥ Ch liposomes ≫ SS liposomes.

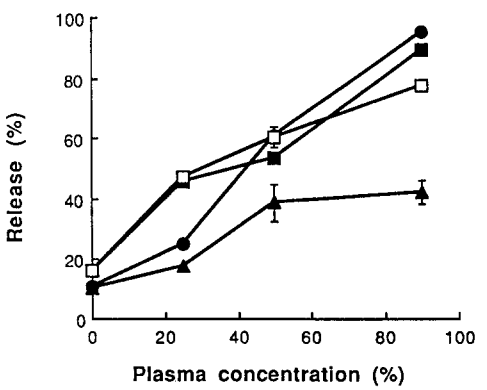


Fig. 3. Effects of rat plasma concentration on the release of calcein from liposomes after incubation at 37±0.5° C for 1 h (DPPC/X = 7:2; X = SS, Ch, SG). Each value represents the mean±S.D. of three mice. DPPC liposomes (●), SS liposomes (▲), Ch liposomes (■), SG liposomes (□).

Fig. 3 shows the effects of rat plasma concentration on the release of calcein from the liposomes after incubation for 1 h. The degree of release of calcein from the liposomes increased with rat plasma concentration. The release profiles of calcein from SG liposomes were similar to Ch and DPPC liposomes in more than 50% plasma. SS liposomes were the most stable.

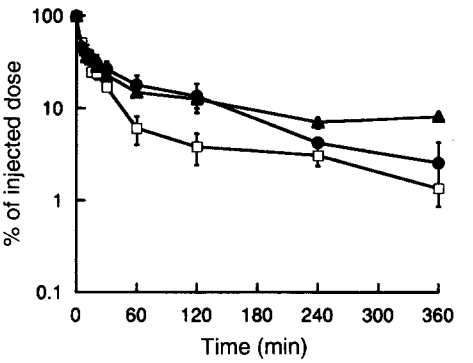


Fig. 4. Decreasing calcein from liposomes in blood after intravenous injection in mice (DPPC/X = 7:2; X = SS, SG). Each value represents the mean±S.D. of three mice. DPPC liposomes (●), SS liposomes (▲), SG liposomes (□).

3.2. Blood circulation

The concentration of calcein entrapped in the liposomes in the blood was expressed as a percentage of the injected dose as shown in Fig. 4. DPPC, SS and SG liposomes were present at about 2.6, 8 and 1.4%, respectively, of the injected dose in the blood circulation at 6 h. The calcein concentration of SS liposomes was higher than those of the other liposomes at 6 h (3–6

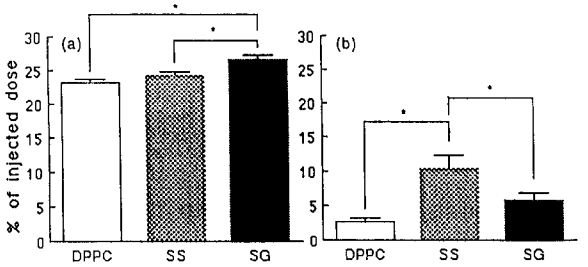


Fig. 5. Biodistribution of liposomes 6 h after intravenous injection in mice. Liver (a) and spleen (b) (DPPC/X = 7:2; X = SS, SG). Each value represents the mean ± S.D. of three mice. * $p < 0.05$.

fold). Liposomes in the blood 6 h after i.v. injection remained greater in the following order: SS liposomes > DPPC liposomes > SG liposomes. However, Ch liposomes were not observed in the blood 5 min after administration.

3.3. Biodistribution and hepatic cellular distribution

In order to clarify the difference in blood circulation time among the liposomes, RES uptake and hepatic cellular distribution of the liposomes were examined. First, the distribution of the liposomes in liver and spleen was investigated.

Fig. 5 indicates the biodistribution of liposomes 6 h after i.v. administration to mice. SG liposomes showed significantly high accumulation in the liver, about 26% of the dose, but only 5% of the dose in the spleen whereas SS liposomes amounted to about 24% of the dose in the liver and 10% of the dose in the spleen.

Fig. 6 demonstrates the cellular distribution of liposomes in the liver 2 h after i.v. administration to mice. DPPC liposomes were not taken up by parenchymal cells and non-parenchymal cells. This result indicates that DPPC liposomes were not stable in plasma and showed little distribution in the liver cells. On the other hand, SG liposomes were also unstable in plasma but were significantly highly distributed in the parenchymal cells compared with the SS and DPPC liposomes.

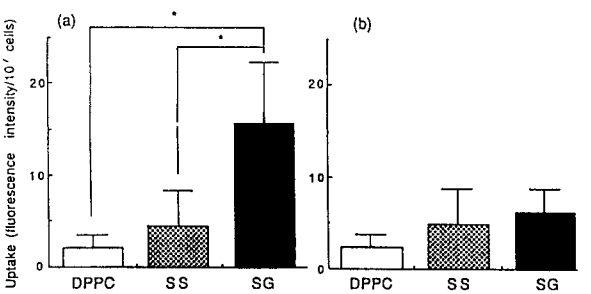


Fig. 6. Hepatic cellular distribution of liposomes 2 h after intravenous injection in mice. Parenchymal cells (a) and non-parenchymal cells (b) (DPPC/X = 7:2; X = SS, SG). Each value represents the mean ± S.D. of three mice. * $p < 0.05$.

4. Discussion

4.1. Stability of liposomes in plasma in vitro

In general, the liposomes were unstable in plasma. Scherphof et al. (1980) have demonstrated that the destabilizing action of liposomes is caused by serum proteins, in particular by high-density lipoproteins, and that cholesterol increases liposomal stability by reducing interactions between lipids and serum proteins. Based on a previous in vitro study (Muramatsu et al., 1994), SS liposomes are expected to be stable in the blood because of the rigidity of the liposomal membrane. It is interesting to investigate the difference in the in vivo characteristics between SS and SG liposomes considering the behavior in vitro. Liposomes of DPPC with Ch, SS or SG in a molar ratio of 7:2 were selected, since up to this ratio SG may be mixed homogeneously in the DPPC liposomes as found for Ch and SS measured by DSC (unpublished data) and fluorescence anisotropy (Muramatsu et al., 1994).

Fig. 2 indicates that the liposomes were destabilized by components in the plasma. To determine the reason, the destabilization of liposomes in plasma was investigated after preheating at 56° C for 30 min (data not shown). The release of calcein from SS liposomes decreased from 33 to 20% and that from SG liposomes from 61 to 45%. The investigators (Finkelstein and Wissmann, 1979) also reported that a reduction in plasma mediated marker release was partially achieved by preheating at 56° C for 30 min.

This result suggested that the liposomes were destabilized by plasma components which were inactivated by heating at 56° C for 30 min. The apolipoprotein has been described as being heat labile (Tall et al., 1976), and it has also been reported that the phosphatidylcholine transfer activity of high-density lipoprotein (HDL) is facilitated by a heat-labile factor in plasma (Senior et al., 1983). In our study, an increase in the stability of liposomes in plasma suggested a decrease in the interaction between the liposomes and proteins in the blood. SS liposomes are stable in the blood; in contrast, SG liposomes appear to be affected by incubation time and plasma concentration (Fig. 2 and 3). This suggests that the plasma proteins may interact with SG liposomes, especially the glucose parts.

4.2. Blood circulation and biodistribution

It is well known that liposomes are rapidly taken up by the RES. In particular, the distribution of liposomes in the liver is connected with the size of the liposomes and the hydrophilicity of the liposomal surface (Namba et al., 1990). Secondly, the composition or rigidity of the liposomes is another important factor affecting their elimination half-life ($t_{1/2}$) in the blood.

Recently, we demonstrated the physicochemical properties of DPPC, SS, Ch and SG liposomes in vitro (Muramatsu et al., 1994). The fluorescence anisotropy values of DPPC, SS, Ch and SG liposomes were determined to be 0.213, 0.255, 0.254 and 0.202, respectively. When the lipids went from a gel to a liquid-crystal state above the transition temperature, the fluorescence anisotropy decreased because the liposomal fluidity increased. The anisotropy values of SS liposomes are greater than those of SG liposomes. SS liposomes have low fluidity compared with DPPC and SG liposomes. Therefore, Fig. 2 and 4 indicate that the low fluidity of SS liposomes in vitro was correlated with the stabilization of the liposomes in plasma and in the blood circulation. On the other hand, DPPC and SG liposomes showed a high fluidity of the lipid layer and were destabilized in plasma in vitro and in the blood circulation. The $t_{1/2}$ values for DPPC, SS and SG liposomes were 85.0, 147.2 and 75.6 min, respectively (Fig. 4). The $t_{1/2}$ and AUC of liposomes were significantly greater in the following order: SS liposomes $\gg$ DPPC liposomes > SG liposomes. SS liposomes show significantly higher AUC and $t_{1/2}$ values than DPPC and SG liposomes.

4.3. Hepatic cellular distribution

It is well established that the RES, principally the Kupffer cells and secondarily the splenic macrophage cells, is responsible for the clearance of liposomes from the circulation. Fig. 5 indicates that each liposome is accumulated in the liver and spleen. SG liposomes showed considerable accumulation in the liver 6 h after intravenous injection. Liu et al. (1992) reported that ganglioside GM_1 containing liposomes (egg phosphatidylcholine/Ch/GM_1 = 10:5:1) was found in the liver (20%) for liposomes with a size of 100 nm in diameter. Our data correspond well with their results. Ch liposomes were not observed in the blood 5 min after i.v. injection.

It is not clear what kind of cells in the liver contributes to the enhancement of hepatic uptake. The liver cells consist of parenchymal and non-parenchymal cells, distinguished by their morphological differences such as cell size.

The data in Fig. 6 further indicate that stable liposomes, SS liposomes, did not accumulate in parenchymal and non-parenchymal cells, whilst SG liposomes accumulated significantly in parenchymal cells. This may suggest that SS induces liposomal membrane rigidity, preventing the liposomes from being opsonized and captured by the Kupffer cells.

Scherphof et al. (1983), Rahman et al. (1982), and Roerdink et al. (1984) reported that small unilamellar liposomes could extravasate and accumulate in the parenchymal cells of liver. This is because the fenestra of the liver sinus contains holes of 100 nm average diameter (Wisse, 1969). In this study, the diameter of the liposomes is about 100 nm, and SS and DPPC liposomes were not accumulated in the parenchymal cells, whereas accumulation of SG liposomes did occur.

Mannose and galactose have often been employed in the targeting of liposomes to Kupffer cells and parenchymal cells, respectively (Leserman and Machy, 1987), however, the use of glucose has not been reported. The results for SG liposomes may suggest that the glucose unit of SG is useful for targeting of the liver in comparison with SS liposomes. This observation suggests that the enhanced uptake of SG liposomes in parenchymal cells results from the facilitation of liposome uptake. However, the mechanism of uptake of SG liposomes by parenchymal cells has not yet been clarified.

Because the glucose unit connects directly to the sterol part of SG, it is suspected that it may project outward from the liposomal surface. By observing the difference between SS and SG liposomes in the blood circulation and hepatic cellular distribution, it could be attributed to the fact that the glucose unit of SG may dominate. Further investigations in this field are continuing.

The natural or synthetic glycolipids have the limitation that they are very difficult to prepare in a highly purified form and are thus very expensive. Although SG is a mixture, it is an abundant product. Therefore, these results are considered to be of considerable practical significance.

5. Conclusions

SS liposomes were more stable than Ch liposomes in vitro and in vivo due to the low fluidity of the liposomal layer. DPPC and SG liposomes were not stable in vitro. SG liposomes showed a short residence time in the blood circulation and an enhancing effect on hepatic uptake different from those of DPPC liposomes. The difference in these characteristics of SS and SG liposomes may be deduced to result from the glucose group of SG, which might project out from the surface of the liposomes. These results are expected to be useful for liposome applications, since SS can be used as a substitute for cholesterol, and SG liposomes can be used for targeting to parenchymal cells in the liver.

Acknowledgements

The authors are grateful to Ms Akiko Sugahara and Mr Hiroki Usui for assistance in the experimental work and to Ms Noriko Sato for help in the collagenase perfusion experiment.

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