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Novel Long-Circulating Liposomes Consisting of PEG Modified β-Sitosterol for Gambogic Acid Delivery

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Long-circulating liposome is an effective formulation in field of cancer treatment. However, high expenditure of formulation and high dose of cholesterol severely restrict its application. In this paper, we developed a method by grafting polyethylene glycol 2000 on β-sitosterol succinic anhydride ester to obtain relatively cheap polyethylene glycol-β-sitosterol conjugates, which were used to prepare long-circulating liposome without cholesterol. Gambogic acid which is an effective anti-tumor ingredient with very short half-life, was used as a model drug to prepare long-circulating liposome in this research. Meanwhile, the characteristics, pharmacokinetics and distribution of this novel long-circulating liposome were also investigated in comparison with other gambogic acid formulations. Polyethylene glycol-β-sitosterol conjugates were synthesized, different liposomal formulations were also prepared by ethanol injection method, and the obtained nanoparticles were characterized by dynamic light scattering and transmission electron microscope. The long-circulating effect, pharmacokinetics and distribution of gambogic acid in rats were also explored. ^{1}HNMR confirmed that polyethylene glycol-β-sitosterol conjugates were synthesized successfully. Novel long-circulating liposome was successfully prepared by ethanol injection method attaining a entrapment efficiency of 89.4%, exhibiting a homogeneous particle size of 245.2 nm and −24.3 mV zeta potential with smooth continuous surface. This novel long-circulating liposome demonstrated better long-circulating effect than ordinary long-circulating liposome. The novel long-circulating liposome as-prepared not only could reduce cost of grafting polyethylene glycol on macromolecular phospholipid, but also no cholestrol in preparation was applied, expanding the application of liposome as a formulation in the field of lowering blood lipid. Therefore, polyethylene glycol-β-sitosterol conjugates are recommended substitute for polyethylene glycol modified phospholipid to prepare long-circulating liposomes.

Keywords: β-Sitosterol, Long-Circulating Liposome, Gambogic Acid, Cholestrol.

1. INTRODUCTION

Liposome was first discovered by Bangham in the mid 60s, now it was regarded as an advantageous candidate in biomedical delivery.[1–7] Further studies showed that long-circulating liposome carriers can provide more advantages, such as, staying in the blood circulation for a relatively long period, delivering passive target drugs into tumors or inflamed tissues where the integrity of the endothelial barrier is destroyed, etc.[8,9] Therefore, long-circulating liposome has become a central theme of research in liposome field. One of the most widely used methods for preparing long-circulating liposome is to apply polyethylene glycol (PEG) modified phosopholipid as a raw material.[10] PEG is considered to form a hydrated shell hindering the interaction of liposomes with serum proteins, thereby greatly reduces the opsonization and uptake by macrophages, which provides a great opportunity for passive targeting of liposomal anticancer agents into tumor tissues because of the capillary permeability of the endothelium in newly vascularized tumors is significantly greater than that of normal organs.[11,12] So far, PEG-modified phospholipids have been mainly utilized as materials to prepare liposomes for anti-tumor therapy due to their long-circulating advantage. However, the expensive PEG-modified phospholipids limit the application of long-circulating liposome because the modification,

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separation and purification of macromolecules is considerably more difficult than small molecules. Both phospholipid and PEG are macromolecules, such as distearoyl phosphoethanolamine (DSPE) and PEG 2000, and could be strongly adsorbed in silicone column which resulted in low yields. Moreover, high dose of cholesterol (Chol) severely restricts the application of liposome because Chol is not suitable for certain people, especially for patients with cardiovascular disease or high Chol disease. Those defects seriously limited the application of the long-circulating liposome in clinic.[13] It was very urgent to explore a novel long-circulating liposome to overcome those shortcomings.

Gambogic acid (GA, Fig. 1), is the major active ingredient of gamboges.[14–18] Recent studies from several groups had demonstrated that GA possessed potent anti-cancer activity both *in vitro* and *in vivo* in animal models.[19–22] However, GA posses a very short half-life, just 15 min after i.v. administration in rat, which retarded the preclinical study and further application.[23, 24]

In our previous research, it was verified that β-sitosterol could be used to institute cholesterol in liposome to prepare no-Chol liposome formulation.[25, 26] In this research, PEG was chemically grafted on relatively cheaper and smaller β-sitosterol succinic anhydride ester (SS) to obtain polyethylene glycol-β-sitosterol (PEG-SS), then PEG-SS were adopted as liposome material to prepare a novel GA loaded long-circulating liposome. Characterization, pharmacokinetics, biodistribution and the long-circulating effect of this novel long-circulating liposome were explored and compared with ordinary long-circulating liposome where PEG modified phospholipid was used as a raw material (GPD).

2. MATERIALS AND METHODS

2.1. Synthesis of PEG-SS Conjugates

The synthetic process of PEG–SS conjugates is displayed in Figure 2 according to the literature.[27] Briefly, β-sitosterol (Aladdin, Shanghai, China), 4-dimethylaminopyridine (DMAP; Aladdin, Shanghai, China) and succinic anhydride (Aladdin, Shanghai, China) dissolved in dichloromethane (DCM) were reacted for 48 h with stirring, thereafter DMAP was removed by washing with HCl solution and saturated $NaHCO_3$ solution. The combined organic layer was dehydrated with anhydrous Na_2SO_4 and then concentrated to achieve SS (Compound 2). Secondly, SS, DMAP, 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC; Aladdin, Shanghai, China) and PEG 2000 dissolved in chloroform were refluxed for 24 h. The mixture was concentrated under vacuum, and then re-dissolved with DCM. The DMAP and other by-products were removed as mentioned in preparation process of SS. PEG–SS was purified on a silica-gel column, eluting with a mixed solvent system of DCM and methanol.

^{1}HNMR spectra of succinic anhydride, β-sitosterol, SS, PEG and PEG-SS were recorded on a AVYIII-500MHz instrument (Bruker, Billerica, MA, USA) at room temperature. All the samples were dissolved in $CDCl_3$.

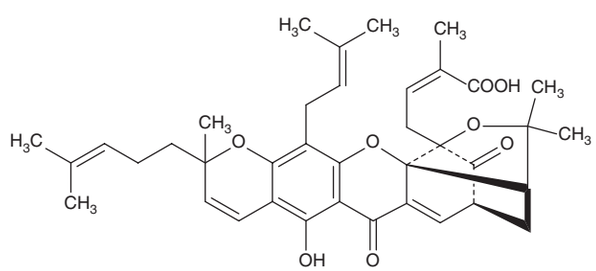

Figure 1. Chemical structure of GA.

2.2. Preparation of Liposomes

Various liposomes were prepared by ethanol injection method.[28] Briefly, GA, Distearoyl Phosphoethanolamine (DSPE; CordenPharma, Plankstadt, Germany) and PEG–SS at different molar ratios were dissolved in ethanol. The solution was injected into a 40 °C PBS (7.0), kept stirring for 2 hours to evaprate ethanol, then filtrated through a 450-nm filter, the GPS liposome was obtained.

Bare liposome was composed of GA, DSPE and β-sitosterol. Ordinary long-circulating liposome (GPD) was composed by GA, DSPE-PEG-OH (Nanocs, Boston, MA, USA) and β-sitosterol. Both of them were prepared by the same method as GPS. Blank liposome, blank GPD and blank GPS were prepared by the same method but without GA. Experiments were performed in triplicate.

Entrapment efficiency of GA in the liposome was calculated using following equation:

$$\text{Entrapment efficiency (\%)} = \frac{\text{weight of GA in liposome}}{\text{weight of GA fed initially}} \times 100\% \quad (1)$$

2.3. Morphology

The morphology of various liposomes was observed by transmission electron microscope (TEM; FEI Tecnai G20, Hillsboro, Oregon, USA) at 200 kV. Sample solution was dropped on the copper grid with a film and dried before observation.[29]

2.4. Dynamic Light Scattering (DLS)

The particle size and zeta potential were measured by DLS using a Malvern Zetasizer Nano-ZS90 (Malvern, Grovewood, UK). All of the DLS measurements were performed at 25 °C.

2.5. HPLC Analysis of GA

Entrapment efficiency of GA was measured using a reverse-phase HPLC method according to Qu's report.[30]

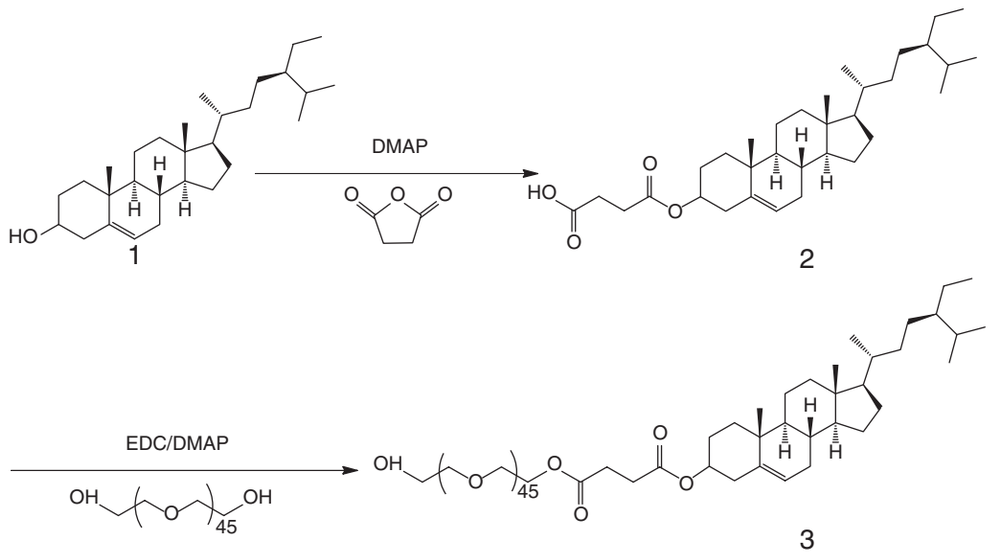

Figure 2. Synthesis scheme.

Briefly, Shimadzu LC-10AD (Shimadzu, Nakagyo-ku, Kyoto, Japan) was used and chromatographic separation was achieved using a Phenomenex column (4.6 × 250 mm, Hanbon, Huaian, Jiangsu, China) at 30 °C. Mobile phase consisted of water and HPLC grade methanol (6:94) was adjusted to pH 3.5 using phosphoric acid. The samples were eluted at a flow rate of 1.0 ml/min and detected at 360 nm using UV detection. The GA amount in sample solution was analyzed after appropriate dilution with methanol, and the injected volume of sample was 20 μL. Otherwise, the GA levels in blood or tissue samples were determined with pretreatment.

2.6. Pharmacokinetic Study

Twenty four Sprague-Dawley rats (180–220 g, Qilongshan Animal Co., Ltd., Nanjing, Jiangsu, China) were used and randomly divided into four groups ($n = 6$). Then the sample solutions were injected into the tail vein of rats at a dose of 4 mg/kg. At 2, 5, 10, 15, 20, 30, 40, 50, 60, 90, and 120 min after injection, blood samples (0.5 mL) were collected; 0.10 ml plasma was obtained by centrifugation at 3000 rpm for 10 min, felodipine (20 μL of a 1 μg/mL solution) was used as internal standard (IS), 50 μL hydrochloric acid solution (1 mol/L) and 0.3 mL acetonitrile were added before vortexing for 3 min. After centrifugation at 10,000 rpm for 10 min, 20 μL of clear supernatant was injected into the HPLC system directly and the GA concentration in rat plasma was calculated by standard curve.[31] The GA plasma concentration over time was analyzed by using the software package program 3P97 (The Committee of Mathematic Pharmacology of the Chinese Society of Pharmacology) and the following pharmacokinetic parameters were obtained: area under the plasma concentration time curve (AUC), plasma half life ($T_{1/2}$), total plasma clearance (Cl), and apparent distribution volume (Vd).

2.7. Tissue Distribution Determination

Thirty-six rats were randomly divided into four groups ($n = 3$). GA-ARG, Bare liposome, GPD and GPS solutions were intravenously administrated via tail vein in a dose of 4 mg/kg. At the time points of 5, 15 and 45 min after injection, rats were sacrificed. The organs (heart, liver, spleen, lung, kidney, and brain) were excised and thoroughly washed with normal saline, then blotted dry and weighed. Subsequently, the weighted tissues were homogenized (BioSpec Products Inc., Tearork, Bartlesville, OK) with 3-fold weight of normal saline. 100 μl of homogenate, 20 μL felodipine (1 μg/ml), 25 μL of hydrochloric acid solution (1 mol/L) and 300 μL of acetonitrile were added into a glass tube. After being vortexed for 3 min, the mixture was centrifugalized at 3,000 rpm for 10 min, and 20 μL of the supernatant was injected into the HPLC system. The concentrations of GA in samples were analyzed under the condition described above.

2.8. Statistical Analysis

Statistical analysis was performed by Student's *t*-test for two groups. A probability (p) of less than 0.05 is considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Synthesis of PEG–SS Conjugates

Figure 3 shows the ^{1}HNMR spectra of succinic anhydride, β-sitosterol, SS, PEG and PEG-SS in $CDCl_3$. The double peaks at δ 5.37 and 5.36 (a) were attributed to the protons of olefinic bond (–CH_2CH=C–) in β-sitosterol. The double peaks at δ 2.31 were attributed to

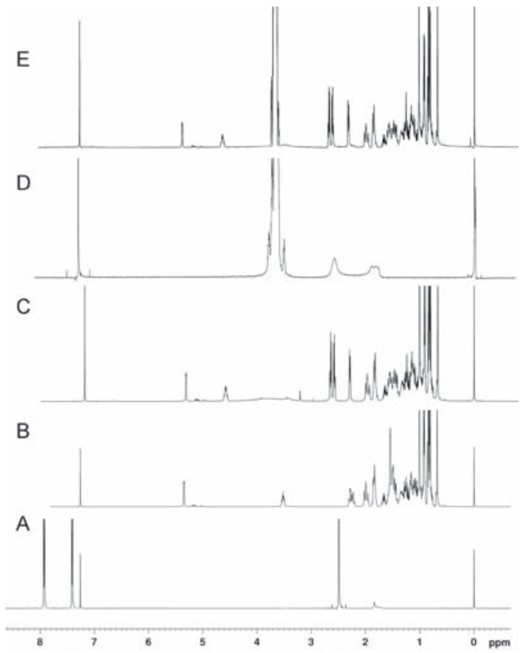

Figure 3. Comparison of the [1]HNMR spectra of succinic anhydride, β-sitosterol, SS, PEG and PEG-SS in $CDCl_3$. ((A), Succinic anhydride; (B), β-sitosterol; (C), SS; (D), PEG; (E), PEG-SS).

the protons of C4 (–CH_2–) in SS. The triplet peaks at δ 2.59 and 2.67 came from the protons of succinate linkage (–(C=O)CH_2–CH_2(C=O)–) in succinic anhydride. The multiple peaks at δ 3.65 were attributed to the protons from the glycol unit (–O–CH_2–CH_2–O–) in PEG chain. Thus PEG-SS conjugates have been successfully synthesized.

3.2. Preparation and Characterization of GPD and GPS

The GA loaded long-circulating liposomes (GPD and GPS) were prepared by a simple ethanol injection method. DLS measurement revealed that the average particle size and zeta potential of blank liposome were 67.3 nm and −28.4 mV (Table I), respectively, while the size and potential of blank long-circulating liposomes were changed greatly, the particle size was increased to 178.5 nm in blank GPD and 156.5 nm in blank GPS, revealed that PEG extended from liposome occupied a certain space.

Table I. Size, zeta potential and entrapment efficiency of different formulations.

Formulations	Size (nm) (PDI)	Zeta potential (mV)	Entrapment efficiency (%)
Blank liposome	67.3 (0.103)	−28.4	–
Bare liposome	114.7 (0.136)	−31.5	92.5
Blank GPD	178.5 (0.119)	−15.7	–
GPD	267.9 (0.124)	−17.2	90.1
Blank GPS	156.5 (0.116)	−21.1	–
GPS	245.2 (0.115)	−24.3	89.4

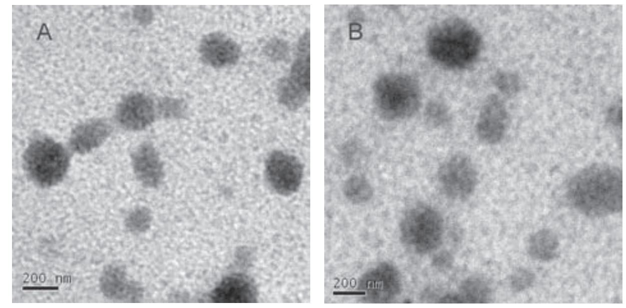

Figure 4. Transmission electron microscope (TEM) images of GPD and GPS liposomes ((A), GPD; (B), GPS).

Meanwhile, the absolute value of potential were decreased to 15.7 mv in blank GPD and 21.1 mV in blank GPS, indicating that the charge shielding effect of electrically neutral PEG encapsulated around liposome, which was reported previoiusly.[32,33] After encapsulation, the particle size increased in all formulations. Bare liposome, GPD and GPS were increased to 114.7, 267.9 and 245.2 nm, suggesting that a larger compact hydrophobic inner cavity was formed after GA loaded. The zeta potential of blank liposome, blank GPD and blank GPS were −28.4, −15.7 and −21.1 mV, respectively. After GA loaded, the zeta potential of them decreased about 3 mV to −31.5, −17.2 and −24.3 mv, suggesting that GA charged negatively slightly decreased zeta potential of formulations, which was consistence with our previous research results.

Entrapment efficiency in the GPD and GPS liposomes were calculated to be 90.1% and 89.4%, the results were closed to 92.5%, which was obtained from ordinary bare liposome group without containing PEG, indicating that the existence of PEG did not significantly influence the encapsulation of GA. The high entrapment efficiency also confirmed that PEG-SS is suitable for GA as a long-circulating liposome carrier.

The representative TEM of GPD and GPS liposomes (Fig. 4) showed that long-circulating liposomes were almost uniform spherical in shape with particle diameter 250 nm, there were not significant difference between GPD and GPS in appearance.

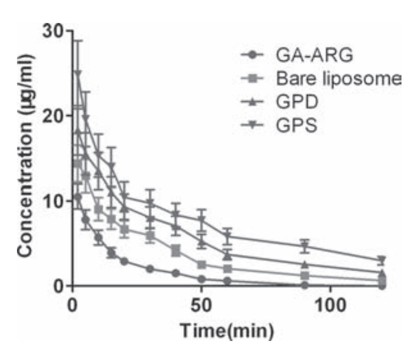

Figure 5. Profile of plasma concentration-time after i.v. four formulations to rats ($n = 6$).

Table II. Pharmacokinetic parameters of four GA formulations in rats after i.v. administration at dosage of 4 mg/kg ($M \pm SD$, $n = 5$).

Formulation	V_d (ml)[a]	$T^{\circ}_{1/2}$ (min)[b]	AUC (Infinite) ($g \times$ min/ml)[c]	Cl (mL/min)[d]
GA-ARG	0.415	14.27	198.56	0.020
Bare liposome	0.426	35.64**	482.69**	0.008**
GPD	0.348*	48.67**	807.32**	0.005**
GPS	0.266**	54.75**	1187.51**	0.003**

Notes: [a]apparent volume of distribution. [b]elimination half-life. [c]area under the plasma concentrationetime curve. [d]total body clearance. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with GA-ARG.

3.3. Pharmacokinetics of GPD and GPS

Liposomes modified with PEG are aimed at obtaining liposomes with long-circulating characteristics: PEG encapsulated the liposome to form a hydrated shell hindering the interaction of liposomes with serum proteins, thereby greatly reducing the opsonization and uptake by macrophages. Therefore, the drug carried by liposomes should be present longer in plasma without being trapped in reticulo endothelial system (RES).[34] To confirm this, rats were injected with GA-ARG, Bare liposome, GPD and GPS via the tail vein. The pharmacokinetics data was obtained after injection indicated those liposomes, including Bare liposome, GPD and GPS, circulated longer in bloodstream compared with GA-ARG solution. The concentration-time curves (Fig. 5) and elimination half-life $T_{1/2}$ (Table II) showed that the half of GA was eliminated within 15 min, this result was consistence with previous report,[31] which indicated that GA is not stable in blood, and is eliminated rapidly from blood circulation. Compared with the GA-ARG group, the $T_{1/2}$ of GA in Bare liposome, GPD and GPS were increased to 35.64, 48.67 and 54.75 min, respectively, increased about 2.5, 3.4, 3.8 times ($p < 0.01$) and the Cl of each formulations was decreased accordingly. Compared with GA-ARG group, bare liposome, GPD and GPS had significant differences with it. The results indicated that nano-system of liposome could prolonged half-life of GA in the body from 14.27 to 35.64 min. Long-circulating liposomes modified by grafting PEG, including GPD and GPS, could even keep liposomes present much longer in blood without being trapped in RES, resulting in further prolongation of their blood circulation time, even if GPS was prepared by PEG-SS, exhibiting much longer long-circulating characteristics than ordinary GPD. However, there was no difference between them in statistics. The longer $T_{1/2}$ of GPS might be attributed to the smaller particle size and lower absolute zeta potential. According to the report,[35] lower absolute zeta potential of liposome system means much longer in blood without being trapped in RES. This result clearly indicated that PEG conjugated on SS could substantially influenced the hepatic handling of liposome as the ordinary liposome modified on phospholipid.[36–39]

Area under the plasma concentration time curve (AUC) of various formulations of GA were shown in Table II. The AUC value of Bare liposome, GPD and GPS were 2.43, 4.07 and 5.98 times higher than that of the GA-ARG ($p < 0.01$). The prolonged half-life, decreased clearance, and comparative AUC contributed to the long-circulating characteristics of liposomes in the systemic circulation. It is hypothesized that the presence of PEG on the surface of liposomes attracts a water shell, resulting in the reduced adsorption of opsonins to the liposomes.[40–42] This, in turn, results in a decrease in both the rate and extent of uptake of liposome by the mononuclear phagocyte system.[43] Smaller nanoparticle size indicated the particle is not easily cleared by the body.[44–46] Compared with ARG-GA, apparent volume of distribution (V_d) of GPD and GPS, significantly decreased to 0.348 and 0.266, indicating

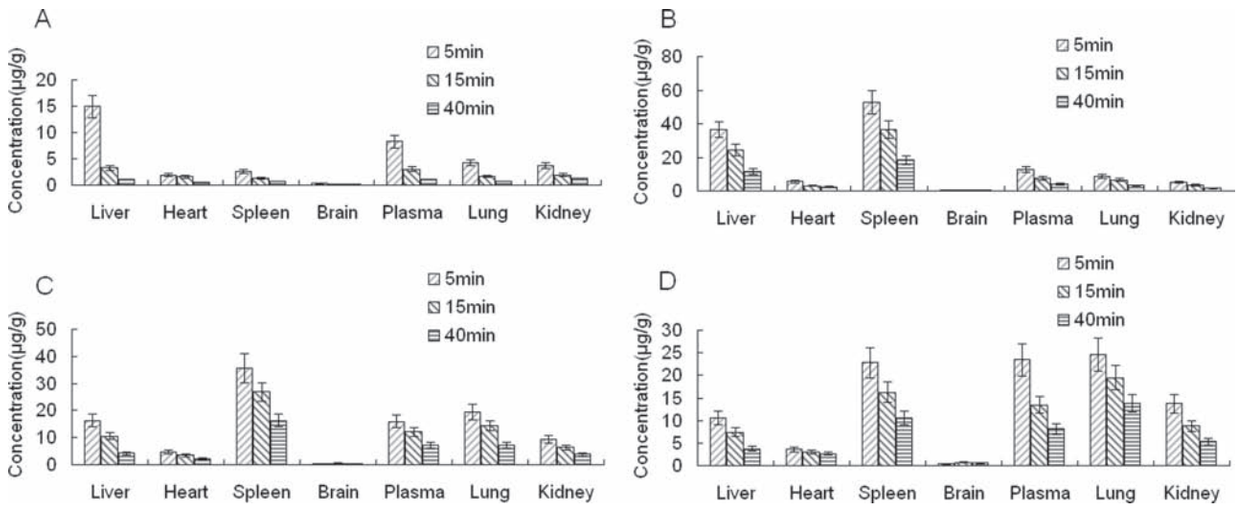

Figure 6. Distributed concentrations of various GA formulations in plasma and tissues at different time by intravenous administration ((A), GA-ARG; (B), Bare liposome; (C), GPD; (D), GPS; $n = 6$).

that the distribution of drug GA decreased, displaced a certain degree of passive targeting.

3.4. Tissue Distribution of Four GA Formulations

The passive targeting characteristics of those drug delivery systems were evaluated by tissue distribution experiment according to the value of concentration of GA in different organs.[47] All the profiles were shown in Figure 6. GA-ARG group was set as control group. In GA-ARG group, GA was concentrated sequentially in the liver, plasme, followed by kidney, lung, spleen, heart, brain, and GA eliminated quickly in each tissue. In bare liposome group, the distribution of GA mainly focused in spleen, liver and plasma, and the concentration of GA in 5 min was increased greatly in comparison with GA-ARG group, there were significant differences between two groups ($p < 0.01$). This might be attributed to the formulation of liposome and could change the delivery of GA *in vivo*. In two long-circulating groups, GPD and GPS group, both of the distribution and concentration of GA were changed greatly in comparison with GA-ARG group. In GPD group, GA mainly focused in spleen, lung, plasma and liver. The concentration of GA was increased greatly in comparison with that of GA-ARG group, there were significant differences between two groups ($p < 0.01$). The reason might be the long-circulating liposomes are not readily taken up by the macrophages in RES, and hence they remain in the blood circulation for a relatively long period of time and transport to rich blood organs, such as spleen, liver, and lung.[48,49] In another long-circulating GPS group, GA mainly concentrated in lung, plasma, spleen, liver. The distribution of GA in GA-ARG was found in the liver, demonstrating a first-pass effect of liver. In liposome groups, including bare liposome, GPD and GPS, the concentrations of GA in liver and spleen were very high, expressed the nano-particle system could passively target the drug to liver, spleen.[50–52] Interestingly, the concentration of GA in spleen were higher than that in liver in liposomal groups. The reason might be that both spleen and liver have a rich blood flow, but enzyme in spleen is less than that in liver. Therefore, the concentration of GA in spleen was higher than that in liver in those three groups.

4. CONCLUSIONS

The purpose present study was to investigate the feasibility of coupling of PEG onto the SS to prepare novel long-circulating liposome and explore its pharmacokinetics, distribution *in vivo*. The results clearly showed that this novel long-circulating liposome was prepared successfully, the results of pharmacokinetics indicated this novel liposome composed of PEG-SS significantly prolonged blood circulation time of GA after intravenous administration *in vivo*. Therefore, the preparation of long-circulating liposome using PEG-SS substituting for PEG modified DSPE is feasible.

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