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Preparation, Characterization, and Evaluation of Liposomal Ferulic Acid In Vitro and In Vivo

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In the present study, various gradients were evaluated for efficient loading of weak acid into liposomes. Several salt gradients showed efficient loading of ferulic acid (FA) into liposomes and the optimized conditions were established in calcium acetate gradient method to obtain 80.2 ± 5.2% entrapment efficiency (EE). Unilamellar vesicles were observed in micrographs and liposomal FA showed good stability. 80% of FA was released from liposomes within 5 h in vitro. There is a novel finding in this study: that drugs could be entrapped with a high solubility in the intraliposomal buffer in contrast to the low solubility in the extraliposomal buffer. The results of body distribution in rats indicated that liposomes could improve the body distribution of FA. For FA liposome, the concentration of FA in brain was two-fold higher than that of free FA. Liposomal FA was a promising approach to improve the body distribution of FA.

Keywords liposome; remote loading; calcium acetate gradient; solubility; body distribution; ferulic acid

INTRODUCTION

Traditional Chinese Medicines (TCMs) are commonly advocated treatments for a wide range of conditions in many Eastern countries and also have become popular in the West (Ming et al., 2006; Song et al., 2002). TCMs now have received extensive attention and intensive research with reference to their low incidence of toxic side effects compared to synthetic drugs (Butterfield, Castegna, Lauderback, & Drake, 2002). Ferulic acid (4-hydroxy-3-methoxycinnamic, FA) is the primary active ingredient in several Chinese medicinal herbs (Boyd-Kimball et al., 2005; Gururaj et al., 2006) and has been used clinically to treat neurovascular and cardiovascular diseases for many years. Previous investigations suggest that FA can exhibit significant pharmacological effects, including antioxidant, radical scavenging, anti-apoptotic, anti-inflammatory, anticancer, antiaging, neuroprotective, and antidiabetic properties (Butterfield, Castegna, Pocernich & et al., 2002b; Ogiwara et al., 2002; Srinivasan et al., 2006). Hence FA is considered a promising protective agent for the treatment of neurodegenerative disorders, such as cerebral ischemia reperfusion, Parkinson's, and Alzheimer's diseases (Kikuzaki et al., 2002; Kim et al., 2004), which are characterized by free radical-mediated oxidative stress, apoptosis, and brain inflammation (Butterfield, Castegna, Pocernich et al., 2002b; Butterfield, Drake, Pocernich, & Castegna, 2001). However, its poor penetration of the blood-brain barrier (BBB) limits the application to neurodegenerative diseases.

The application of liposomes as delivery systems has received attention and research for the advantage of reducing the toxicity and improving the distribution and therapeutic effects of drugs in organisms. In the past two decades a number of methods, including pH gradient (Du & Deng, 2006; Santos et al., 2004) and ammonium salt gradient (Haran, Cohen, Bar,

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& Barenholz, 1993), were developed to enhance EE (entrapment efficiency). Stéphane Clerc (Clerc & Barenholz, 1995) described a novel method for remote loading amphipathic weak acid into preformed liposomes. In both concepts of remote loading, the drugs form insoluble salt complexes in the liposomal interior (Clerc & Barenholz, 1995; Haran et al., 1993). Our findings, however, were inconsistent with the previous reports.

In the present study, further gradients were evaluated for efficient loading of weak acid into liposomes. Several salt gradients showed efficient loading of FA into liposomes. The liposomes via calcium acetate gradient exhibited 80.2 ± 5.2% EE for FA. Then the characterization of liposomal FA was evaluated by average size, stability, release of FA from liposomes in vitro, and body distribution in rats. The results indicated that liposomal FA exhibited satisfying properties in vitro and in vivo with a significant increase in concentration in the brain.

MATERIALS AND METHODS

Materials

Soybean phosphatidylcholine (SPC) was obtained from Taiwei Pharmaceutics Corp. (Shanghai, China). Cholesterol (Ch) was of analytical grade from Tianjin Chemical Reagent Co., Inc. (Tianjin, China). Sephadex G-50 was from Pharmacia. Ferulic acid (FA) and salicylic acid (SA) were products of Wanma Synthetic drug Corp. (Zhejiang, China). IL-1β was product of Pierce (USA). Dialysis bags were from Sigma (USA). All the other chemicals were of analytical grade.

Preparation of Liposomes

SPC (Soybean phosphatidylcholine, SPC > 99.4%) and Ch (Cholesterol, > 99.8%) (at 1.54 molar ratios) were dissolved in about 5 ml of chloroform and dried in a rotary evaporator under reduced pressure (Hwang et al., 2004). The dried lipid film was re-dissolved in 1 ml of chloroform and mixed with 10-fold various dilution solutions (Table 1). Each mixture was subjected to bath-type sonication and vortexed to obtain a homogeneous w/o emulsion. The emulsion was then placed on a rotary evaporator under reduced pressure for at least 12 h to ensure the remove of organic solvent. Then the suspension was placed in an ice-bath and subjected to 500 watt of ultrasonic treatment for 2 min using a high-intensity probe ultrasonicator (JY92-2D; Xinzhi Equ.Inst., China).

Preparation of Salt Gradient

The salt gradients were prepared via size exclusion chromatography. The liposome suspensions were eluted with Sephadex G-50 (20–80 μm) column pre-equilibrated with 120 mM Na_2SO_4 solution (pH 6.3). Thus, Na_2SO_4 acted as the extraliposomal phase for all these salt gradients. Subsequently FA was added into liposome suspensions at 5:1 lipid-to-drug molar ratio and incubated with the liposomes under nitrogen protection at 37°C for 10 min.

For calcium acetate gradient, the influence of different formulation factors and process parameters including cholesterol content, lipid-to-drug molar ratio, incubation temperature, and time on the EE were investigated.

Determination on Entrapment Efficiency (EE)

HPLC and ultraviolet spectrophotometry were both used to determine the concentration of FA. The equipments for HPLC were a mobile phase delivery pump (LC-10AD; SHIMADZU, Japan) and a UV–VIS detector (SPD-10A; SHIMADZU, Japan). A C_{18} reverse-phase column (Thermo Quest Hypersil® ODS2 column, 150 mm × 4.6 mm, 5 μm) and a Phenomenex C_{18} security guard (4 mm × 3.0 mm, 5 μm, Torrance) were used to separate FA with the mobile phase of acetonitrile (chromatographic grade)-0.02% glacial acetic acid (chromatographic grade) (20:80). Spectrophotometer UV9100 (Beijing, China) was used for spectrophotometry. The equation for calculating the EE was as follows:

$$EE = W_{interior}/W_{total} \times 100\%$$

Where $W_{interior}$ represented the intraliposomal content of FA and W_{total} represented the total content in the liposomal suspension when Triton-100 was added to the suspension.

Measurement of Saturated Solubility of FA in Intraliposomal and Extraliposomal Solutions

FA was added in excess to 10-milliliter solvents (including sodium sulfate solution and those solvents shown in Table 1) to ensure saturation at 50°C. Sonication was used to accelerate dissolution. Then, the systems were left to equilibrate for 72 h at 25°C. Then, the samples were centrifuged and filtered. The concentrations of FA in the supernatants were determined to calculate saturated solubility in different solvents.

TABLE 1
The Various Solutions for Internal Phase of Liposomes

	$Ca(Ac)_2$	$Mg(Ac)_2$	NaAc	KAc	$CaCl_2$	NaCl	$NaHCO_3$	Na_2CO_3
pH	7.0	7.3	7.6	7.9	7.2	7.6	8.3	10.9
Concentration (mmol/l)	120	120	150	150	120	150	150	150

Study on Average Size and Zeta Potential of FA Liposomes

The internal/external phases of liposomes for average size and zeta potential assay were as follows: H_2O/H_2O, $Ca(Ac)_2/Na_2SO_4$. The average diameter of liposomes was determined with dynamic laser light scattering methods using Coulter LS-230 (Beckman, USA). The zeta-potential was measured by Nicomp-38ZLS Zeta Potential equipment (Santa Barbara, USA). Two ml of liposome suspensions were diluted with 100 ml freshly distilled water for average size and zeta potential determination at room temperature.

Stability Study

FA liposomes via calcium acetate gradient method were stored in 10 ml ampoules filled with nitrogen at 4°C. The EEs and average size of samples were determined during six months to estimate the stability.

Freeze-Fracture Electron Microscopy

The freeze-fracture electron was employed as described elsewhere (Sternberg, 1993), and a freeze-fracture device (Balzers BAF 400D) was used for fracturing. The replicas were detected under the transmission electron microscope. The samples of blank liposome and FA liposomes for freeze-fracture electron microscopy were prepared via calcium acetate gradient.

Drug Release Experiments

Liposomes via acetate salt gradient were passed through Sephadex G-50 to remove the free FA and obtain samples for release experiment of FA liposomes. Dialysis bags were soaked in PBS for 24 h before used. Five ml of samples were poured into dialysis bags. The medium for release experiment was PBS (pH 7.4) and sink condition was ensured. The release of liposomal FA was determined from the measurement of FA in the release medium at 37°C with gentle stir.

Body Distribution Studies

In in vivo studies, the concentration of FA was determined by HPLC as described above using salicylic acid as internal standard. Male Wistar rats (12 weeks, 250 ± 20 g) provided by China Medical University Animals Center were divided randomly into two groups of six rats each. (The experiments complied with the requirements of the National Act on the use of experimental animals, People's Republic of China.) All the rats were fasted overnight but were free to water before experiments. Animals were subjected to intrastriatal microinjections of 100 unit of human recombinant IL-1β in order to produce brain inflammation (Anthoy et al., 1997). Subsequently, two formulations including FA solution and FA liposome (equivalent 5 mg FA/kg body weight) were administration through caudal vein injection.

Determination of FA in Serum

Animals were sacrificed by decapitation after 15 min of administration. Blood was collected into heparinized tubes with 10 μl of internal standard (salicylic acid), and separated immediately by centrifugation (4000 rpm for 15min). One ml of 5% trichloro acetic acid and 1 ml of acetoacetate were added to 150 μl of serum and were mixed by vortexing for 30 s. The mixture was then centrifuged (3000 rpm for 15min). The extract procedure was repeated with another 1 ml acetoacetate. The organic phase was collected and blown to dryness under Nitrogen protection. 200 μl of mobile phase was used to redissolve the residue for determination of FA by HPLC.

Drug Determination in Different Organs

Different organs (heart, liver, spleen, lung, kidney, and brain) were washed with PBS, dried, and weighted. Every organ sample was homogenized and treated in similar manner of serum. Whole organ was homogenized in case of less than 1.0 g.

Statistical Analysis

All results and data were confirmed with at least three independent experiments. The data were expressed as means ± *SD* Statistical comparisons were made by Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

The Effects of Different Salt Gradients on Entrapment Efficiency

The EEs of liposomes obtained from different salt gradients are shown in Figure 1. The EE declined in the line of salt gradients: $Ca(Ac)_2 > Mg(Ac)_2 > NaAc > NaHCO_3 > KAc > CaCl_2 > NaCl > Na_2CO_3$ at 37°C for 10 min incubation. The result indicated that transmembrane gradient of acetate salt generated a more efficient driving force to load FA into liposome. As described in the previous study (Clerc & Barenholz, 1995), the permeability coefficients of acetic acid and of calcium ion differ by seven orders of magnitude. Thus, while calcium ions with lower permeation remain trapped inside the liposomes, acetic acid molecules behave as proton shuttle. In response to calcium acetate concentration differences across the membrane, a net transfer of protons occurs from the inside of the liposomes to the external side, resulting in changes of pH in both compartments for remote loading.

To obtain high EEs, the influence of different formulation factors and process parameters, including cholesterol content and lipid-to-drug ratio, temperature and time of incubation on the EEs were investigated for the calcium acetate gradient method. As shown in Figure 2, the EEs increased with the rise of cholesterol content when the molar ratio of Ch: SPC was below 0.5 (lipid-drug ratio 2.6, at 55°C for 30 min incubation).

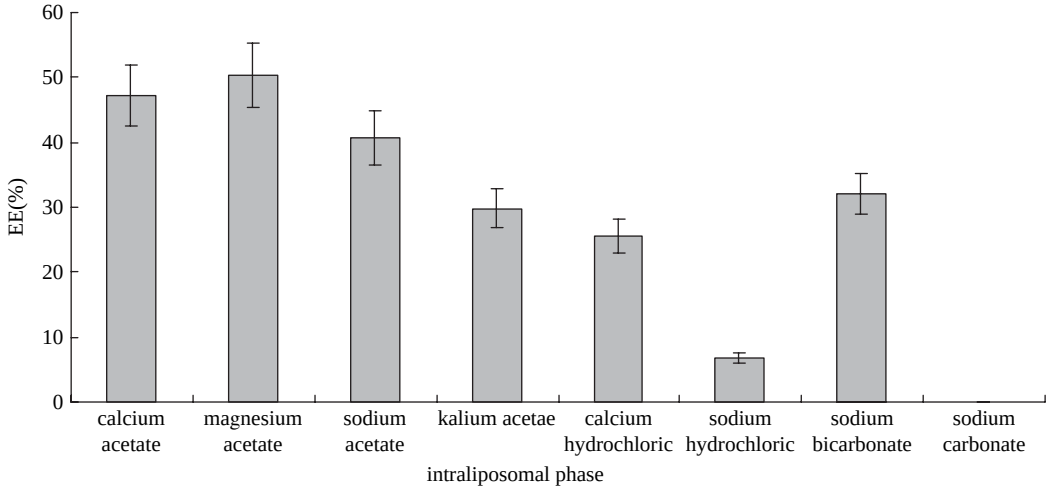

FIGURE 1. The EEs of liposomes via various salt gradients with sodium sulfate as external phase at 37° C for 10 min incubation.

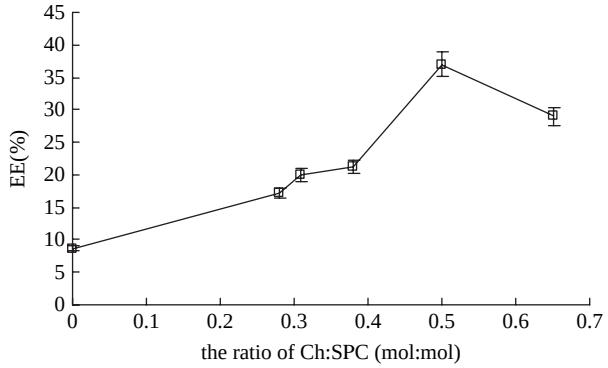

FIGURE 2. The influence of Ch content on EEs at 55°C for 30 min incubation.

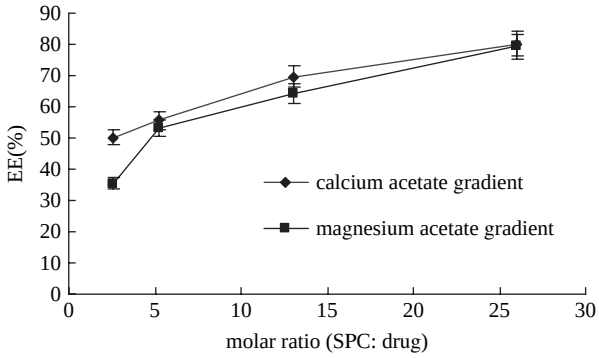

FIGURE 3. The influence of the lipid-to-drug molar ratio on EEs for calcium/magnesium acetate gradient at 37° C for 30 min incubation.

There was a decrease in EEs when the molar ratio of Ch: SPC was above 0.5. It indicated that there was an optimal ratio of Ch: SPC to obtain a sufficiently stiffened membrane of liposome (Sulkowski, Pentak, Nowak, & Sulkowska, 2005).

Incubation time and temperature were negatively related when the temperature was below 55°C. The incubation condition of 37°C, 30 min could contribute to higher EEs. The EEs of liposomes incubated at 37°C for 10 min was equivalent to that incubated at 4°C for 30 min. The modest incubation conditions could not only contribute to remote loading of FA, but also avoid leakage of calcium ion, which acts as a reservoir inside liposomes to sustain the salt gradient across the membrane for longer periods (Clerc & Barenholz, 1995).

As shown in Figure 3, the molar ratios of lipid-to-drug had significant effects on EEs. The EEs increased with the rise of lipid-to-drug molar ratio. The optimized conditions were established to achieve 80.2 ± 5.2% EE with the molar ratios of lipid-to-drug 26:1 and Ch-SPC 1:2, at 37° C for 30 min incubation.

The Saturated Solubility of FA in Intraliposomal Solution

As shown in Figure 4, the solubility of FA in intraliposomal solutions was higher than those in extraliposomal solutions. This was different from the previous study. It is believed that the intraliposomal precipitation maintained the soluble drug gradient, which allowed continuous drug loading (Fritze et al., 2006). In this study, these salt gradients with intraliposomal high solubility could make the drug uptaken into liposomes without drug precipitation. One could assume that the difference in solubility in the two compartments was a synergistic, rather than a decisive, effect on remote loading. High solubility inside liposome also might act as a reservoir with high capacity to keep FA inside liposome.

The Characteristics of FA Liposome

A representative freeze-fracture electron micrograph of blank liposomes and FA liposomes is shown in Figure 5. Large unilamellar vesicles were observed in micrographs.

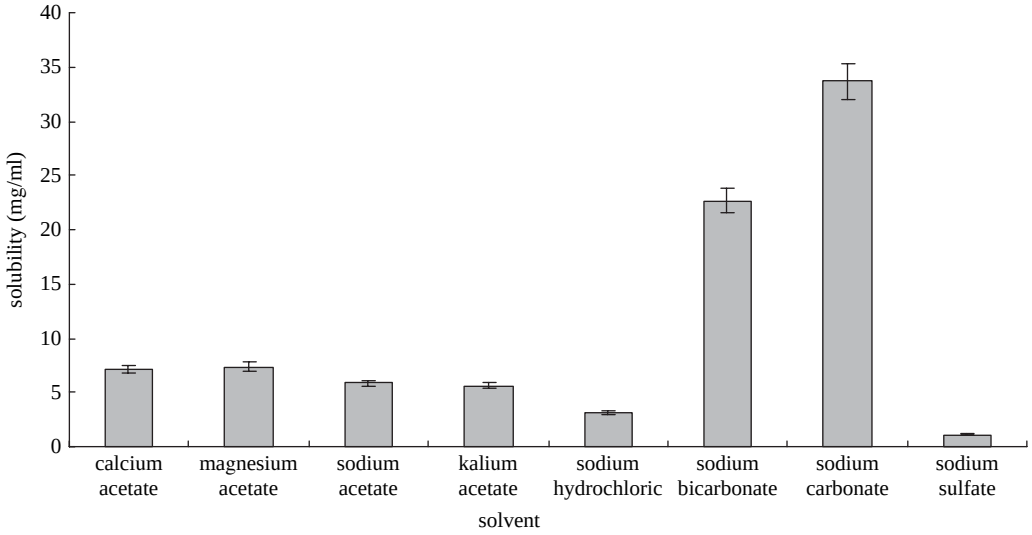

FIGURE 4. The saturated solubility of FA in various solutions for salt gradients.

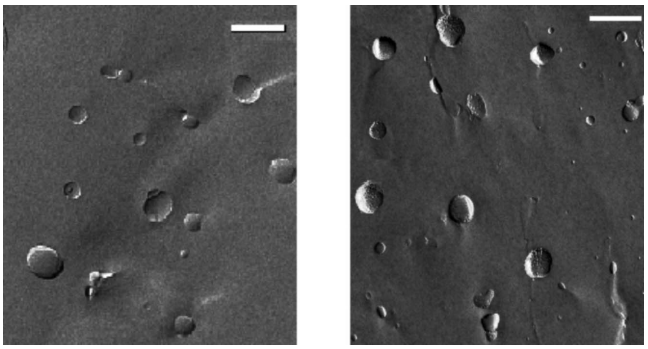

FIGURE 5. Freeze-fracture electron micrograph of blank liposomes (A) and liposomes loaded with FA (B) by calcium acetate gradient. Scale bars represent 200 nm for both A and B.

The average size of net liposome (internal/external phase: water/water) and liposomes via salt gradient were 140 ± 1.14 nm and 152 ± 1.21 nm, respectively. The size of net vesicle was smaller than that of the vesicle of salt gradient. Obvious increments in zeta potential were observed in presence of salt gradient (zeta potential 11.4) compared to net vesicle (zeta potential −10.07). These indicated that the salt gradient significantly affected the mean size and zeta potential. It was confirmed (Ruso et al., 2003) that an adsorption of cations onto the liposomes occurred in the present of cations. The adsorption of cations onto the liposomes might account for the increase in mean size and zeta potential.

There was no significant change in EEs or average size of the liposomal FA during six months (data not shown), which meant aggregation of liposomes did not occur and calcium acetate gradient was stable during six months. It might be due to the low permeation of calcium ion that acted as a reservoir for sustaining the salt gradient across the membrane for longer periods of time (Clerc & Barenholz, 1995). The adsorption of cation onto lipid bilayers might be another reason for the stability of liposomes.

The Release of FA from FA Liposomes

Drug release from liposome was a critical property. The influences of different acetate salt gradients on the release of drug from liposomes were investigated. The release medium would be encountered following their application, physiological pH 7.4 at 37° C. As shown in Figure 6, three acetate salt gradient exhibited similar release profiles and about 80% of FA released from liposomes within 5 h. The pressure derived from high concentration inside liposomes might offer the force for drug release. Furthermore, another reason accounting for the release of FA from liposomes might be that PBS took the place of sodium sulfate that had low permeation coefficient and maintained the same osmolality on both side of the lipid bilayers.

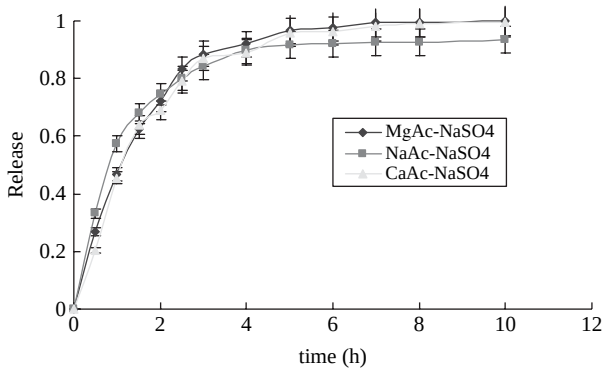

FIGURE 6. The release profiles of FA liposomes via acetate salt gradients.

Body Distribution

Inflammatory response plays an important role in the pathogenesis of neurodegenerative diseases such as ischemia in the brain, Alzheimer's, and Parkinson's diseases (Chopp et al., 1994; Clark, Madden, Rothlein, & Zivin, 1994; Gendeiman et al., 1994). Accumulating evidences show that IL-1β, a proinflammatory mediator, contributes to the postischemic intracerebral inflammatory response and neurological damage (Huang & Tatro, 2002; Touzani, Boutin, & Chuquet, 1999; Yang et al., 1999). IL-1β dramatically increases after ischemia, and stimulates the release of cytokines that can aggravate brain injury following postischemic reperfusion (Rezzonico, Chicheportiche, & Imbert, 2000; Wang, Wu, & Huang, 2004). Hence, an injection of IL-1β into the brain parenchyma could produce an inflammatory response in brain within 4 h (Blamire et al., 2000), which has good correlation with disease conditions.

Figure 7 shows the FA concentration in different organs after iv administration of different formulations. As shown in Figure 7, free FA was mainly accumulated in kidney at the FA concentration of 19.0 ± 1.93 μg/g. For FA liposome, there was a significant decrease in FA kidney concentration (8.5 ± 0.82 μg/g). Then, an increase in FA concentration for liposomal FA was found in liver and spleen that might be due to the absorption by reticuloendothelial system (RES). Liposomal FA (1.35 ± 0.04 μg/g) could be mediated into the brain with a 2-fold concentration compared to free FA (0.65 ± 0.03 μg/g).

It was found that liposome encapsulated antioxidants penetrate cells at a much faster rate than the free antioxidants (Michelson & Puget, 1980). Furthermore, as described by Sinha (Sinha, Das, & Basu, 2001), the antioxidant-loaded liposomes were found to be greatly potent compared to free drug in controlling the conjugated diene increment in the brain of rats after ischemia and reperfused rats. The treatment of L-ascorbic acid encapsulated in liposomes was 4.5 times more effective than an identical amount of free antioxidants in reducing the diene concentration of rat brain. But liposome with the lipid mixtures without any antioxidant was ineffective to resist the diene elevation in brain caused by ischemia and reperfusion. In this study, the high concentration in brain of liposomal FA compared to free FA was consistent with the previous findings. Hence, liposomal FA might be a promising strategy for treatment of brain diseases with regard to the significant antioxidant, radical scavenging and anti-inflammatory properties.

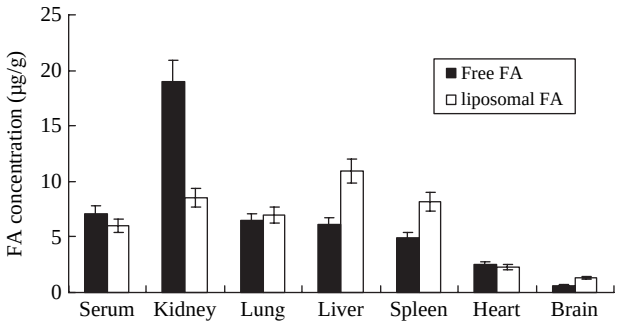

FIGURE 7. The results of body distribution in rats of FA solution and liposomal FA after iv administration 15 min.

CONCLUSION

In the present study, further gradients were evaluated for remote loading of weak acid. Several salt gradients showed efficient loading of FA into liposomes. The optimized conditions of formulation factors and process parameters were established for calcium acetate gradient with 80.2 ± 5.2% EE. Liposomal FA via salt gradient exhibited a good stability during six months. The results of saturated solubility of FA in various solvents indicated that high EEs could be obtained via intraliposomal high solubility that also might act as a reservoir for remote loading to keep FA inside liposome. Furthermore, about 80% of FA released from liposomes within 5 h. The results of body distribution in rats showed that liposomal FA exhibited a higher FA concentration in the brain than FA solution. Hence, liposomal FA might be a promising approach to improve the body distribution and therapeutic effects of FA for the treatment of neurodegenerative disorders.

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