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Alpha-Asarone incorporated in mixed micelles suitable for intravenous administration: formulation, *in-vivo* distribution and anaphylaxis study

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Alpha-Asarone is clinically used as a commercial intravenous formulation (CA-AREs). However, Polysorbate 80, a solubilizing agent contained in the formulation has been reported to be toxic. To enhance the aqueous solubility of ARE and to reduce the toxicity of CA-AREs caused by solubilizing agents, ARE loaded soybean phosphatidylcholine-deoxysodium cholate-mixed micelles (ARE-SPC-DC-MMs) were prepared and characterized in this study. Furthermore, pharmacokinetics and tissue distributions of ARE-SPC-DC-MMs and CA-AREs were also investigated. Additionally, the anaphylaxis of both of the two formulations was evaluated. The mean size of mixed micelles (ARE-SPC-DC-MMs) was 24.74 ± 1.14 nm and the ARE solubility within the mixed micelles was approximately 30-fold greater than that of free drug in water. ARE-SPC-DC-MMs showed pharmacokinetic parameters similar to CA-AREs. AUC $_{(0\rightarrow t)}$ (mg/L·min) of ARE-SPC-DC-MMs was lower in spleen than that of CA-AREs ($p < 0.05$) while it was greater in the lung ($p < 0.05$). There was no significant difference in other organs. These findings demonstrated that in comparison with CA-AREs, ARE-SPC-DC-MMs had similar properties *in vivo*, but led to higher accumulation of ARE in lungs. Meanwhile there was nearly no anaphylactic reaction caused by ARE-SPC-DC-MMs, but reactions could be observed in the CA-AREs group with significantly higher histamine release. In conclusion, ARE-SPC-DC-MMs could be an excellent substitute for commercially available CA-AREs for intravenous administration.

1. Introduction

Alpha-Asarone (ARE, 2, 4, 5-trimethoxypropenylbenzene) (Fig. 1) is the effective ingredient extracted from *Acorus gramineus* Soland. It also can be synthesized and has been used clinically for the treatment of pneumonia, asthma and grand mal (Zhao et al. 2007; Xu et al. 2007; Antunez-Solis et al. 2009). The application of ARE is limited due to its poor water-solubility and low bioavailability (Wang et al. 2008; Guo et al. 2008). Hence intravenous administration is the most preferred route for ARE. In order to solve the problems described above, CA-AREs, a commercially available intravenous single micelles formulation, has been produced by solubilizing ARE with the simple surfactant Polysorbate 80 and applied clinically in China for decades (Wu et al. 2010). However, a good deal of noticeable allergies, aroused within 10 min, has always existed in the clinic especially for children, of which the anaphylactic shock occupies 51.5% incidence (Wu et al. 2010). Due to its fast responses and severe harms, the allergies are considered to be extremely risky because patients may die from the respiratory failure if the treatment is delayed. It has been reported that Polysorbate 80 contained in CA-AREs may cause anaphylactic reactions (Luo et al. 2010; Zhang et al. 2008). In addition, the increased release of histamine caused by Polysorbate 80 has been demonstrated to be the mechanism of inducing anaphylaxis (Masini et al. 1985; Varma et al. 1985).

Therefore, it is necessary and urgent to develop a safe injectable formulation for ARE by pharmaceutical approaches. To avoid

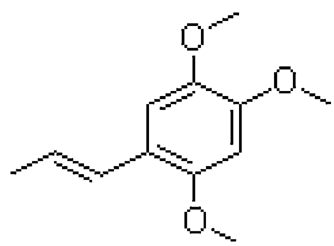

Fig. 1: Chemical structure of alpha-asarone

the use of Polysorbate 80, other dosage forms were adopted in the intravenous application, such as lipid emulsions (Guo et al. 2008). However, the result of tissue distribution studies showed that lipid emulsions increased the uptake of alpha-asarone in liver and spleen, which was unsatisfactory because the increased accumulation of drug in these organs might reduce its efficacy. In order to reduce the uptake of ARE in liver or spleen and enhace its uptake in other organs, micelle formation was considered to be a promising technique.

As solubilizing vehicles for some poorly soluble drugs, phosphatidylcholine-bile salts mixed micelles (PC-BS-MMs) have been prepared through a simple and convenient way (coprecipitation method) (Hammad and Müller 1998a, b, 1999; Son and Alkan-Onyuksel 1996; Duan et al. 2011). As for phosphatidylcholine, soya and egg and even hydrogenated phosphatidylcholine are usually employed (Hammad and Müller

Table 1: Mean size, PDI (polydispersity index) and solubilization of ARE-SPC-DC-MMs and CA-AREs (n = 5)

Samples	Mean size (nm)	PDI	Solubilization (mg/ml)
CA-AREs	11.88 ± 0.02	0.055 ± 0.01	4.00[a]
ARE-SPC-DC-MMs	24.74 ± 1.14	0.074 ± 0.02	4.71 ± 0.16

[a] The value means the labeled ARE amounts of CA-AREs.

1998a; Rupp et al. 2010). Bile salts with a hydrophobic hydrocarbon surface and a hydrophilic surface produced by polar hydroxyl and carboxy groups (Narain et al. 1998) are present in bile in high concentrations and are critically important for the absorption of lipids from the intestine. The hemolytic effect of bile salts could be neutralized by the solubilized phospholipids (Narain et al. 1998; Salvioli et al. 1993; Moschetta et al. 2000). In addition, it has been proved that PC-BS-MMs were locally and systemically tolerated and no embryotoxic, teratogenic or mutagenic effects were observed after administration of PC-BS-MMs (Teelmann et al. 1984). Besides, PC-BS-MMs have also been utilized for the delivery of amphotericin B (Dupont 2002) and vitamin K (Soedirman et al. 1996).

Therefore the objective of this study was to develop a substitute with better quality for CA-AREs. Firstly, ARE loaded PC-BS-MMs were produced and characterized. Subsequently the pharmacokinetics and tissue distributions of ARE loaded soybean phosphatidylcholine-deoxysodium cholate-mixed micelles (ARE-SPC-DC-MMs) and the commercially available alpha-asarone solutions (CA-AREs) were studied and compared using rats and mice as animal models respectively. Additionally, the anaphylaxis of the two formulations was tested in guinea pigs because of their innate sensitivity.

2. Investigations, results and discussion

2.1. Preparation and characterization of ARE-SPC-DC-MMs

The formulation was optimized by a single factor test and the optimal one was made of 12 mg of ARE, 50 mg of SPC, 80 mg of DC and 2.5 ml of saline. Table 1 shows the characterization results of the optimal formulation of ARE-SPC-DC-MMs as well as CA-AREs. The solubility of ARE in water (4.71 ± 0.16 mg/ml) after formation of ARE-SPC-DC-MMs was significantly increased compared to free ARE (about 0.10 mg/ml according to the reference (Wang et al. 2009). Furthermore its solubility in water was higher than that of CA-AREs. The size of CA-AREs was very small (11.88 ± 0.02 nm) indicating that micelles might also be formed in CA-AREs as Polysorbate 80 could form micelles when its concentration was greater than CMC (critical micelle concentration). The characterization results suggested that the produced ARE-SPC-DC-MMs were comparable with CA-AREs.

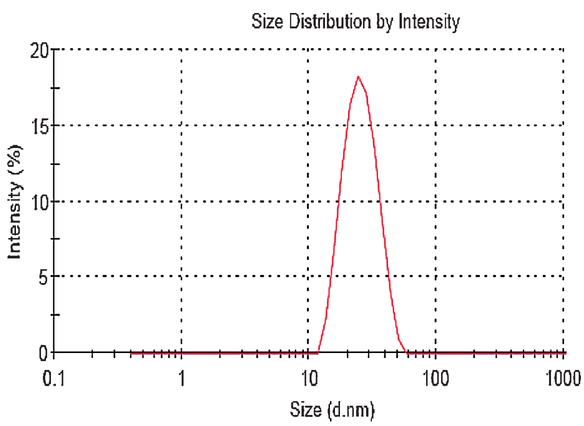

Fig. 2: Size and size distribution of ARE-SPC-DC-MMs

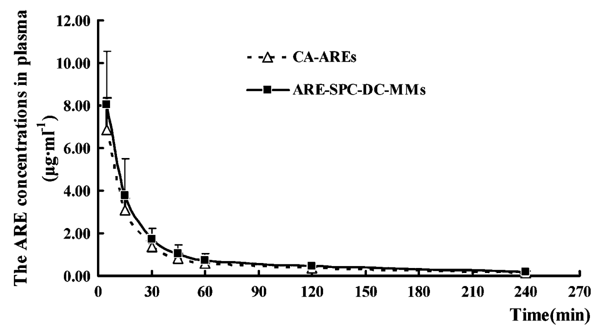

Fig. 3: ARE concentration-time profiles in plasma after intravenous injection of ARE-SPC-DC-MMs and CA-AREs (n = 5)

2.2. Pharmacokinetics

The mean plasma concentration-time curves of ARE after intravenous administration of ARE-SPC-DC-MMs and CA-AREs to rats showed that the elimination of ARE from blood was rapid up to 30 min followed by the slow elimination phase in both of the preparations (Fig. 3). The pharmacokinetic parameters were calculated by a kind of DAS software and the results are shown in Table 2. The AUC and C_{max} of ARE-SPC-DC-MMs were a little higher than that of CA-AREs but the MRT and CL_z were similar. The reason why ARE-SPC-DC-MMs were rapidly cleared after intravenous injection was assumed that ARE-SPC-DC-MMs probably binded to the apolipoprotein quickly followed by rapid clearance from plasma due to the hydrolysis of lipoprotein lipase (Shigeru et al. 2000).

2.3. Tissue distribution study

The distribution results of ARE in each tissue and blood at 5, 15, 30 and 60 min after intravenous injection of ARE-SPC-

Table 2: Noncompartmental pharmacokinetic parameters of ARE after tail vein injection of ARE-SPC-DC-MMs and CA-AREs to rats (n = 5)

Parameters	ARE-SPC-DC-MMs	CA-AREs
$AUC_{(0\text{-}t)}$ (mg/L·min)	257.796 ± 93.333	203.444 ± 20.083
$AUC_{(0\text{-}\infty)}$ (mg/L·min)	287.899 ± 128.664	229.740 ± 26.420
$MRT_{(0\text{-}t)}$ (min)	46.180 ± 4.360	42.919 ± 11.137
$MRT_{(0\text{-}\infty)}$ (min)	75.093 ± 29.258	78.354 ± 21.943
C_{max} (mg/L)	8.052 ± 2.488	6.876 ± 1.502
$t_{1/2z}$ (min)	85.186 ± 33.167	95.364 ± 24.611
CL_z (L/min/kg)	0.079 ± 0.027	0.088 ± 0.011

The data are mean ± SD (n = 5); AUC, area under the concentration-time curve; MRT, mean residence time; C_{max}, maximum plasma concentration; $t_{1/2z}$, half-life; CL_z, clearance.

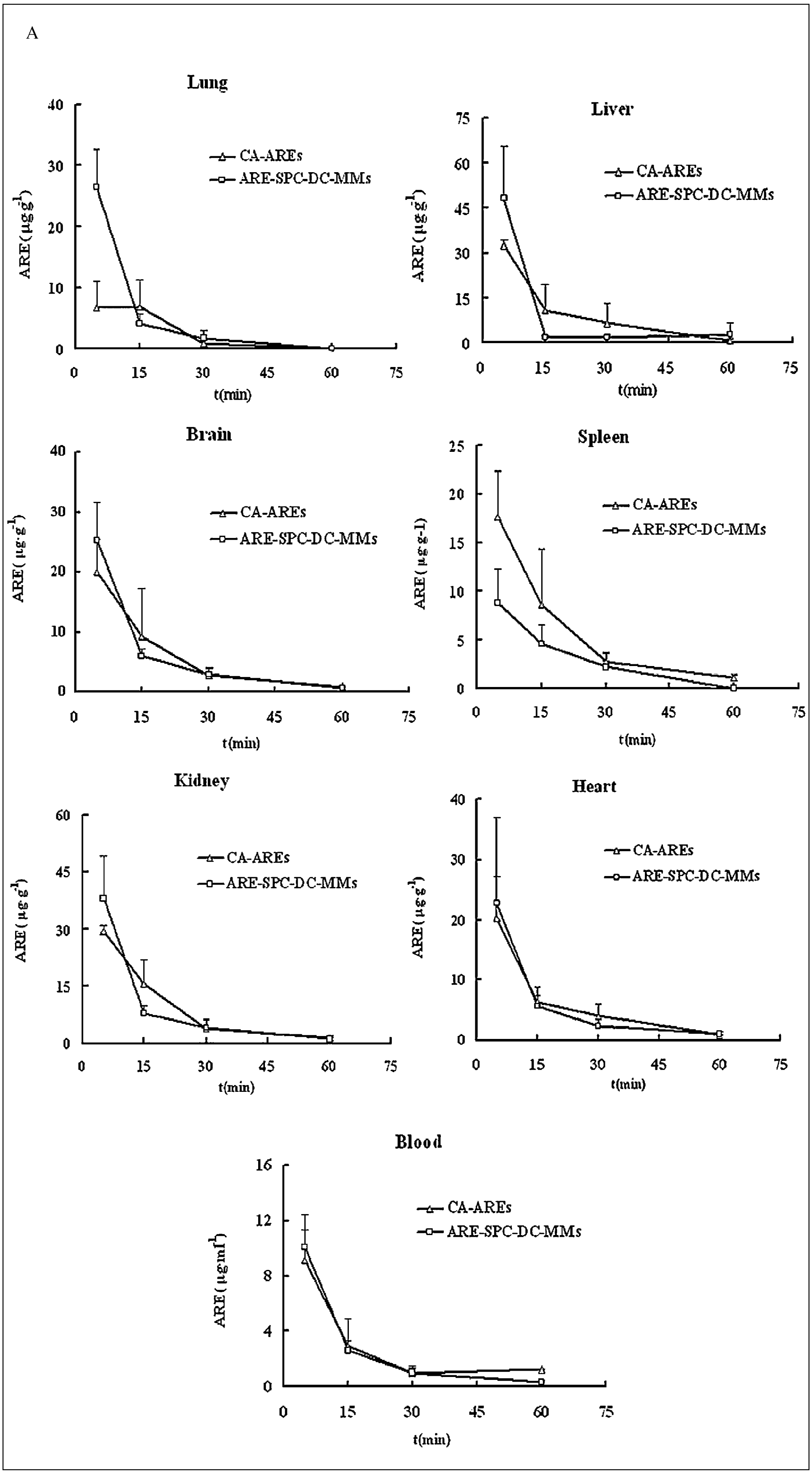

Fig. 4A: . The ARE concentration of ARE-SPC-DC-MMs and CA-AREs in tissue at successive times (5, 15, 30 and 60 min) following i.v. administration to mice at single 40 mg/kg dose of ARE (n = 5)

DC-MMs and CA-AREs are shown in Fig. 4. There was no significant difference in distribution between the two formulations in liver, brain, kidney, heart and blood, respectively. The distribution results in blood were consistent with the results of the pharmacokinetics study. It was interesting, that the distribution of ARE for ARE-SPC-DC-MMs group in lung was

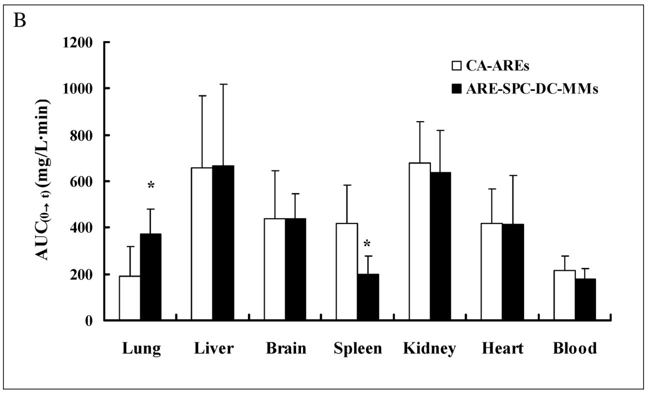

Fig. 4B: . AUC (mg/L·min) for ARE-SPC-DC-MMs and CA-AREs in plasma and tissues of normal mice for 60 min (n = 5). *$p < 0.05$ versus the corresponding organs in the CA-AREs group

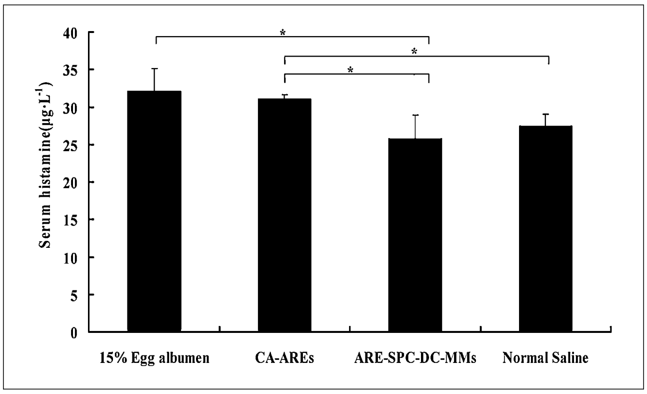

Fig. 5: Serum histamine value obtained after challenging in the anaphylaxis study (n = 4). *$p < 0.05$

significantly higher than that of CA-AREs group ($p < 0.05$), but lower accumulation in spleen for ARE-SPC-DC-MMs group was observed ($p < 0.05$). It was assumed that in comparison with CA-AREs, ARE-SPC-DC-MMs could relatively reduce the uptake by the reticuloendothelial system (RES). The enhanced distribution in lung might be due to the strong interaction between deoxysodium cholate and the lung surfactant dipalmitoylphosphatidylcholine (DPPC) (Mohapatra and Mishra 2010; Subuddhi and Mishra 2007). Additionally, the higher accumulation in lung might be beneficial to the therapy of lung diseases, such as pneumonia.

2.4. *Anaphylaxis study*

In case of ARE-SPC-DC-MMs group, 0.43 ml of ARE-SPC-DC-MMs was provided for sensitization and 0.86 ml for challenge. That's because the ARE content of 0.43 ml or 0.86 ml ARE-SPC-DC-MMs was equivalent to that in 0.5 ml or 1.0 ml CA-AREs (Table 1).

Generally, when an animal is intraperitoneally injected with an immunogen, a strong IgE response is induced, and a subsequent challenge is producing an allergic response. Once the challenge injection was given intravenously, an immediate hypersensitive response might occur with the release of histamine. Tachypnea, expiratory dyspnea, and even spasm were usually observed in the anaphylactic reactions and death would occur due to the overreactions (Daheshia et al. 2001; Lee et al. 1996).

In this anaphylaxis study, allergy symptoms (such as gait disorder and spasm) were extremely obvious in the positive control group, and all animals in this group died within five minutes after the challenge injection. In the CA-AREs group, syncope and gait disorder were observed after the challenge injection. As expected, the behavior of animals in ARE-SPC-DC-MMs group and the normal saline group was the same.

Histamine, one of the major mediators of immediate hypersensitivity, is ubiquitously distributed in mammalian tissues. Mast cells and basophils have also been identified as histamine contained cells and histamine would be released when these cells were challenged with an allergen (Masini et al. 1985; Okunuki et al. 2000; Ruster et al. 2004; Guo et al. 2001). Therefore the amount of histamine released into the blood after challenging could be used to quantify the immediate hypersensitivity reactions.

The histamine levels of different groups are summarized in Fig. 5. It is obvious that after the challenge injection the content of serum histamine in the positive control group and CA-AREs group was significantly greater than that in the normal saline group and ARE-SPC-DC-MMs group ($p < 0.05$). The histamine release in ARE-SPC-DC-MMs group was reduced by 17% compared to the CA-AREs group. However, there was no statistically significant difference between the ARE-SPC-DC-MMs group and the normal saline group.

The quantified results were consistent with the animals behavior described above. Probably the release of histamine in the ARE-SPC-DC-MMs group was lower because there was no Polysorbate 80 in the formulation. Furthermore there is no report about sensitization induced by SPC-DC-MMs, which was also verified in this present study. Therefore ARE-SPC-DC-MMs could be a promising substitute for CA-AREs with lower histamine release and thus no sensitization.

Based on the above, ARE-SPC-DC-MMs exhibited pharmacokinetics and tissue distribution patterns similar to the commercially available ARE solutions. Meanwhile, there was nearly no anaphylactic reactions caused by ARE-SPC-DC-MMs. The results also indicated that ARE-SPC-DC-MMs appeared to be an advantageous and suitable intravenous dosage form, and a promising substitute for CA-AREs that contain the toxic agent Polysorbate 80. Additionally, due to their simple and convenient preparation technology, ARE-SPC-DC-MMs were probably expected to be industrialized.

3. Experimental

3.1. *Materials and animals*

Soybean phosphatidylcholine (SPC) was purchased from Shanghai TaiWei pharmaceuticals industry Co Ltd (China). Deoxysodium cholate (DC) was purchased from Beijing Aoboxing Biotechnology Co Ltd by shares (China). Alpha-Asarone (99.7% of chemical purity) was a product of Harbin SanLian pharmaceuticals industry Co Ltd (China). The commercially available alpha-asarone solutions (Batch number: 090303) were purchased from GuiLin NanYao pharmaceuticals industry Co Ltd by shares (China). Indometacin was purchased from HuZhou KangQuan pharmaceuticals industry Co Ltd (China). All other chemicals were of HPLC or analytical reagent grade and used directly without further purification. Guinea pig Histamine (HIS) ELISA Kit (Lot: 201101) was purchased from R&D company (USA).

Wistar rats, Kunming mice and guinea pigs were all purchased from Laboratory Animal Center, Sichuan University (China).

3.2. *Preparation and characterization of ARE-SPC-DC-MMs*

With good solubility in anhydrous ethyl alcohol, the drug alpha-asarone was incorporated into SPC-DC-MMs in the way of coprecipitation (Hammad and Müller 1998a; Duan et al. 2011). Briefly, ARE, SPC and DC of different weight ratio were dissolved in anhydrous ethyl alcohol. A film was formed after removal of the organic solvent by evaporation under vacuum at 40 °C. The resulting film was re-dispersed in a certain volume of a dispersion medium (water or normal saline). The unloaded drug was separated by filtration through a millipore filter (pore diameter of 0.22 μm), and the filtrate was collected as the final transparent micellar solution. Particle size was determined by dynamic light scattering at 25 °C using a Malvern Zetasizer Nano ZS90 instrument (Malvern instruments Ltd., UK).

Table 3: The scheme of the anaphylaxis study

Group	Sensitisation			Challenge
	Day1	Day3	Day5	Day15
Positive control group (15%Egg albumen)	IP[a]	IP[a]	IP[a]	IV[b]
CA-AREs	IP[a]	IP[a]	IP[a]	IV[b]
ARE-SPC-DC-MMs	IP[a]	IP[a]	IP[a]	IV[b]
Normal Saline	IP[a]	IP[a]	IP[a]	IV[b]

[a] IP = intraperitoneal administration.
[b] IV = an injection into the vein at the lateral of crus curvilineum.

3.3. Pharmacokinetics of ARE-SPC-DC-MMs after intravenous injection

Ten male wistar rats with weight of 180 ± 20 g (Laboratory Animal Center, Sichuan University, China) were randomly divided into 2 groups (5 rats per group) and fasted overnight with free access to water before experiment. At a dose of 20 mg ARE/kg the two groups were administered with ARE-SPC-DC-MMs and CA-AREs by intravenous injection through tail vein, respectively.

At predetermined time intervals (5, 15, 30, 45, 60, 120 and 240 min) after intravenous injection, 0.3 ml blood was drawn from the ocular vein and put into heparinized tubes. After centrifugation (4,000 rpm, 10 min), 100 µl of collected plasma was stored at −20 °C until further analysis by HPLC.

3.4. Tissue biodistribution of ARE-SPC-DC-MMs after intravenous injection

Forty male Kunming mice (Laboratory Animal Center, Sichuan University, China) with a weight of 17–25 g were randomly divided into 2 groups (20 mice per group) and fasted overnight with free access to water before experiment. At a dose of 40 mg ARE/kg the two groups were administered with ARE-SPC-DC-MMs and CA-AREs by intravenous injection through tail vein, respectively.

At predetermined time intervals (5, 15, 30 and 60 min) after intravenous injection the blood was collected as much as possible through orbit and put into heparinized tubes followed by execution of mice. After centrifugation (4,000 rpm, 10 min), 100 µl of collected plasma was stored at −20 °C until further analysis. Then, liver, spleen, heart, kidney, brain and lung were removed and washed immediately with normal saline to remove the blood remaining on the surface of organs. The weight of each organ was measured and homogenized with normal saline at a ratio of 2 ml/g. Afterwards 0.1 g of each homogenate was subjected to analysis by HPLC.

3.5. Determination of ARE in homogenate or blood samples by HPLC

The content of ARE in homogenate or blood was determined by a reversed-phase HPLC method (Tsai et al. 1992). Briefly, 0.1 g of homogenate or 100 µl of plasma was added to a centrifuge tube and mixed with 10 µl of internal standard (indometacin in methanol at a concentration of 20.4 µg/ml) by vortex mixing for 2 min. Subsequently, 200 µl of acetonitrile was added into the tube followed by vortex for 5 min to extract ARE. The resultant mixture was centrifuged at 12,000 rpm for 5 min and 20 µl of the obtained supernatant was subjected to HPLC analysis. The HPLC system (LC-10A, Shimadzu, Japan) with a reversed-phase column (C18, 250 mm × 4.6 mm, 5 µm) plus a security guard column (C18, 10 mm × 4 mm, 5 µm) (Phenomenex, USA) was adopted. Column temperature was set at 35 °C. The mobile phase was a mixture of double distilled water with methanol at a volume ratio of 25:75. The pH value of the mobile phase was adjusted to 3.9–4.6 using phosphoric acid. The injection volume for samples and standards were 20 µl, the flow rate was 1.0 ml/min, and the detection wavelength was 313 nm.

3.6. Anaphylaxis study

Guinea pigs (220–250 g) were raised in a dedicated area with restricted access and the room temperature was maintained at 25 °C. Standard rat chow and water containing 100 mg/ml of vitamin C were provided. Sixteen guinea pigs were randomly divided into 4 groups (four for each): positive control (15% egg albumen, the fresh egg albumen was purchased from the nearby market and diluted to 15% with the normal saline), normal saline group, CA-AREs group and ARE-SPC-DC-MMs group.

The anaphylaxis study was designed as shown in Table 3 (n = 4) according to the previous literatures (Okunuki et al. 2000; Daheshia et al. 2001). Animals in positive control group were sensitized with 0.5 ml of 15% egg albumen by intraperitoneal injection on days 1, 3, 5 and challenged with an intravenous injection at the lateral of crus curvilineum with 1 ml of 15% egg albumen on day 15. The normal saline group and CA-AREs group were sensitized and challenged in the same way as above with normal saline and CA-AREs solution, respectively. The ARE-SPC-DC -MMs group was provided with 0.43 ml of ARE-SPC-DC-MMs for sensitization and 0.86 ml of ARE-SPC-DC-MMs for challenge.

All groups were observed by eyes in the following 30 min after challenges. Afterwards, 0.5 ml intracardiac blood of every animal was collected into tubes. The blood was left to coagulate at room temperature for 30 min and subjected to centrifugation at 3,000 rpm for 30 min, of which the supernatant serum was collected and the histamine level was determined by ELISA kit (Lot: 201101, R&D company, USA) according to the instruction provided by the manufacturer.

3.7. Statistical analysis

The pharmacokinetic parameters were calculated by a DAS software. Statistical analysis was performed by Student's t-test for two groups. All results were expressed as the mean ± standard deviation (SD). A probability value (p) less than 0.05 was considered statistically significant.

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