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Preparation and Evaluation of 2-(Allylthio)Pyrazine-Loaded Lipid Emulsion with Enhanced Stability and Liver Targeting

Jun-Hee Jang and Chong-Kook Kim
Laboratory of Excellency for Drug and Gene Delivery, College of Pharmacy, Seoul National University, Seoul, South Korea

Han-Gon Choi and Jung Hoon Sung
College of Pharmacy, Yeungnam University, Gyongsan, South Korea

To develop 2-(allylthio)pyrazine (2-AP)-loaded lipid emulsion for parenteral administration, various lipid emulsions were prepared with soybean oil, lecithin, and other carriers using homogenization method, and their physical stabilities were investigated by measuring their droplet sizes. The pharmacokinetics and tissue distribution of 2-AP in lipid emulsion after intravenous administration to rats were evaluated compared with 2-AP in solution. 2-AP was lipophilic, sparingly water-soluble, and unstable in aqueous medium. The 2-AP-loaded lipid emulsion composed of 1% of 2-AP, 4% of soybean oil, 4% of lecithin, and 91% of water was physically and chemically stable for at least 8 weeks. It gave significantly faster clearance of 2-AP and higher affinity to the organs, especially the liver, compared with the 2-AP in solution, suggesting that it could selectively deliver 2-AP to the liver. Thus, the lipid emulsion with soybean oil and lecithin could be used as a potential dosage form with the liver-targeting property and enhanced stability of sparingly water-soluble 2-AP.

Keywords 2-(allylthio)pyrazine; lipid emulsion; stability; pharmacokinetics; liver-targeting

INTRODUCTION

2-(Allylthio)pyrazine (2-AP), a synthetic pyrazine derivative with an allylsulfur moiety (Figure 1), has a binding affinity to cytochrome P450 2E1 and hepatoprotective effects against toxicants (Kim et al., 1997). This material is synthesized from 2-chloropyrazine in two steps. 2-Chloropyrazine was reacted with sodium hydrosulfide in dimethylformamide (DMF) to afford the desired pyrazine-2-thiol, which was coupled with allylbromide in the presence of triethylamine, followed by purification using vacuum distillation to give 2-AP as a clear yellow oil. It is under preclinical development in Yu-Han Pharmaceutical Co. Ltd. (Seoul, South Korea). Thus, there are yet no stability documents by International Conference on Harmonization (ICH) guideline. It was effective in suppressing P450 2E1 expression in vivo, and it enhanced the activities of several enzymes involved in the detoxification of carcinogens (Ha & Kim, 1998; Kang, Ha, Kim, Kim, & Kim, 2001; Kim, Kwak, & Kim, 1999). The chemoprotective effect of 2-AP might be due to the inhibition of cytochrome P450 and the induction of phase II detoxifying enzymes. For example, the specific activities of glutathione S-transferase (GST) and the expression of microsomal epoxide hydrolase were elevated in the liver with increases in their mRNA levels (Kim et al., 1999; Kim, Kim, & Kwak, 1994). Cellular glutathione (GSH) levels were also enhanced by 2-AP (Kim et al., 1997). 2-AP appeared to be an efficacious inducer of major GSTs including rGSTA2, rGSTA3, rGSTA5, rGSTM1, rGSTM2, and glucuronyl transferases (Kim et al., 1999; Kwak, Lee, Kim, & Lee, 1998). With these suggested mechanisms, the various efficacy of 2-AP were investigated with the doses of 10–50 mg/kg, and their dosage forms were 40% polyethylene glycol solutions by peroral or parenteral routes mainly once a day. 2-AP inhibited nitric oxide production by inducible nitric oxidesynthase (iNOS) in lipopolysaccharide-treated animals, which raised the possibility that 2-AP regulates inflammatory responses (Kim et al., 1997). 2-AP inhibited the liver toxicity induced by multiple treatments with dimethylnitrosamine, which is primarily activated by CYP2E1. It significantly reduced the plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), and gamma glutamyl transpeptidase (γ-GT) activities increased by dimethylnitrosamine, and it reduced the dimethylnitrosamine-induced liver fibrosis (Kang et al., 2001). Multiplicities of skin tumors formed in female Institute of Cancer Research (ICR) mice treated with vinyl carbamate or vinyl carbamate oxide were inhibited by treatment with 2-AP, and it also inhibited the mutagenicity of vinyl carbamate in the Salmonella-microsome assay (Surh et al., 1998). 2-AP inhibited the formation of presumptive preneoplastic lesions in the liver of rats exposed to

Address correspondence to Chong-Kook Kim, Laboratory of Excellency for Drug and Gene Delivery, College of Pharmacy, Seoul National University, San 56-1, Shinlim-Dong, Gwanak-Ku, Seoul 151-742, South Korea. E-mail: ckkim@plaza.snu.ac.kr

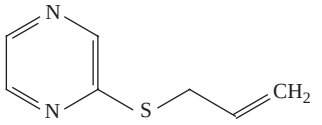

FIGURE 1. Chemical structure of 2-AP.

aflatoxin B_1 (Ha Jang, Kim, & Kim, 1999). Furthermore, it prevented the hepatic toxicity caused by acetaminophen or carbon tetrachloride in animal and significantly reduced the mortality rate induced by toxicants (Costa et al., 2007; Kwak et al., 1998). The hepatic first-pass effect of 2-AP was approximately 20% after oral administration of 10–50 mg/kg to rats, and the gastric first-pass effect contributed considerably to the low extent of oral bioavailability (19.6% at 10 mg/kg) in rats (Han & Lee, 1999). Six metabolites of 2-AP were identified in rat urine (Kim et al., 1997). 2-AP is under preclinical study as a chemoprotective agent to prevent the development of cancer. However, due to the low solubility and oily property of 2-AP, it has been difficult to be administered parenterally without high amounts of surfactants such as polyethylene glycol, which can result in hemolysis.

In this study, to develop 2-AP-loaded lipid emulsion for parenteral administration, various lipid emulsions were prepared with soybean oil, lecithin, and other carriers using homogenization method, and their physical stabilities were investigated by measuring the droplet sizes. The pharmacokinetics and tissue distribution of 2-AP in lipid emulsion after intravenous administration to rats were evaluated compared with 2-AP in solution. Lipid emulsion composed of oil and phospholipid has been applied to drug delivery system for enhancing the solubility, decreasing the toxicity, and increasing the stability of poorly water-soluble drug (Tamilvanan, 2004).

MATERIALS AND METHODS

Materials

2-AP (≥98.5%) was obtained from Yu-Han Pharmaceutical Co., Ltd. (Seoul, South Korea). Lecithin (soybean lecithin), castor oil, soybean oil, and ethyl oleate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogenated castor oil (HCO60®) and medium chain triglyceride (Neobee M5®) were obtained from Gattefosse (Saint-Priest Cedex, France) and Stepan Co. (Maywood, NJ, USA), respectively. All other chemicals were of reagent grade and used without further purification.

Solubility of 2-AP in the Medium

For the determination of solubility of 2-AP, excessive amounts of 2-AP (about 1 g) were added to 1 mL (or 1 g) of water, soybean oil, Neobee M5, ethyl oleate, and castor oil and various pH solutions (pH 1–13, phosphate-buffered saline dry powder blend from Sigma, Dorset, UK), respectively. They were shaken in water bath for 2 days, centrifuged at 5,000 × *g* for 10 min and filtered through membrane filter (0.45 μm) (Yong et al., 2004). The concentrations of 2-AP in the resulting solutions were analyzed by high-performance liquid chromatography (HPLC) (Hitach, Tokyo, Japan) equipped with an Inertsil® ODS-3 C_{18} column (GL science, 0.5 μm, 15 × 0.46 cm i.d.; Tokyo, Japan) and UV detector (Model L-4200; Hitachi). The mobile phase was acetonitrile and distilled water (55:45, volume ratio). The eluent was monitored at 330 nm with a flow rate of 1.5 mL/min (Han & Lee, 1999).

Preparation of 2-AP-Loaded Lipid Emulsion

2-AP was dispersed in oils, such as soybean oil, Neobee M5, ethyl oleate, and castor oil, respectively, and mixed with lecithin and vortexed for 5 min. They were added to water, homogenized at 24,000 × *g* for 5 min using IKA Ultra-Turrax (Ultra-Turrax T 25, Janke & Kunkel, IKA-Lab ortechnik, Staufen, Germany), resulting in isotonic aqueous solution for injection. This procedure was carried out in 4°C to prevent the possible degradation of 2-AP due to heat via vortexing and homogenization (Hwang, Lim, Park, & Kim, 2004; Von, Thoren, & Engstrom, 1998).

To evaluate the physical stabilities of lipid emulsions, their average droplet sizes were measured using a laser droplet size analyzer (Nicomp 770, Santa Babara, CA, USA) (Constantinides & Yiv, 1995).

Pharmacokinetics and Tissue Distribution

Male Sprague–Dawley rats weighing 280 ± 20 g were fasted for 24 h prior to the experiments but allowed free access to water. Twelve rats were divided into two groups. The rats in each group were intravenously infused for 1 min with 2-AP solution (2-AP in 40% PEG 200 aqueous solution) or 2-AP-loaded lipid emulsion (50 mg/kg of 2-AP) after filtering through membrane filter (0.45 μm), respectively. The 2-AP-loaded lipid emulsion was composed of 1% of 2-AP, 4% of soybean oil, 4% of soybean lecithin, and 91% of water (wt/vol). All animals care and procedures were conducted according to the *Guilding priciples in the use of animals in toxicology*, as adopted by the Society of Toxicology in 1999.

Pharmacokinetics

Each rat, anesthetized in an ether-saturated chamber, was secured on a surgical board in the supine position with a thread. A polyethylene tube was inserted into the right femoral artery of the rat, all of the incision was covered with wet cotton, and the cannula was flushed with 0.2 mL of heparinized normal saline (80 U/mL) to prevent blood clotting. 2-AP in 40% PEG 200 aqueous solution and 2-AP-loaded lipid emulsion (equivalent to 50 mg/kg of 2-AP) were infused intravenously for 1 min via femoral vein through the cannula. Half milliliter

of blood was collected from the right femoral artery at various intervals and centrifuged at 3,000 × *g* for 10 min using a centrifuge 5415C (Eppendorf, Hamburg, Germany) (Kwak et al., 1998). Plasma (0.2 mL) was mixed with 0.4 mL of acetonitrile solution containing diazepam (1 μg/mL), as an internal standard. It was then centrifuged at 4,000 × *g* for 10 min to precipitate the proteins. The supernatant layer (0.4 mL) was evaporated under N_2 (*g*). The residue was reconstituted in 50 μL of mobile phase. The resulting solution was then analyzed by HPLC by the previous method (Han & Lee, 1999).

Data Treatment

The noncompartmental pharmacokinetic parameters, such as area under the drug concentration–time curve (AUC), biological half-life ($t_{1/2}$), mean residence time (MRT), total clearance (CL), and apparent volume of distribution at steady state (*V*ss), were calculated based on the reported method (Gibaldi & Perrier, 1982). Levels of statistical significance ($p < .05$) were assessed using the Student *t* test between the two means for unpaired data. All results are expressed as mean ± *SD* values.

Tissue Distribution

2-AP in 40% PEG 200 aqueous solution and 2-AP-loaded lipid emulsion (50 mg/kg of 2-AP) were infused intravenously for 1 min via jugular vein of rats, and rats were sacrificed at 1 h. Blood were taken and treated as mentioned above. Liver, heart, kidney, and spleen were taken, rinsed with ice-cold saline, and weighed. Each sample (0.2 g or 0.2 mL) was mixed with 0.6 mL saline and 0.4 mL of acetonitrile solution containing diazepam (1 μg/mL), homogenized, and centrifuged at 3,000 × *g* for 15 min using a centrifuge 5415C (Yim et al., 2003). The supernatant layer (0.4 mL) was analyzed by HPLC as mentioned above (Han & Lee, 1999).

RESULTS AND DISCUSSION

The physicochemical properties of 2-AP were investigated. 2-AP is an oily brown liquid. 2-AP has a boiling point of 99.05°C (measured by differential scanning calorimetry (DSC), Dupont 9900; Wilmington, DE, USA, data not shown), partition coefficient (log *P*) of 1.94, and aqueous solubility of 3.2 ± 0.4 mg/g, indicating that it was lipophilic and sparingly water-soluble. Aqueous solubility of 2-AP (4–6 mg/mL) showed no significant difference in various pH (pH 1–13), whereas the solubility of 2-AP in oil such as soybean oil, Neobee M5, ethyl oleate, and castor oil was 578.0 ± 44.8, 471.7 ± 35.1, 456.6 ± 19.8, and 571.4 ± 23.4 mg/g, respectively. Among oils tested, 2-AP was more soluble in soybean oil and castor oil. Thus, to prepare 2-AP-loaded lipid emulsion, soybean oil was selected as a carrier, because soybean oil is widely used in emulsion formulations (Driscoll, 2006).

To investgate whether the ratio of oil to lecithin affected the changes of droplet sizes of lipid emulsions, 2-AP (10 mg/mL)-loaded lipid emulsions with the ratio of soybean oil to lecithin of 1:1 and 5:1 were prepared, respectively, and their droplet sizes were measured for 8 weeks (Figure 2). In these emulsion systems, 2-AP (10 mg/mL)-loaded lipid emulsions with the ratio of soybean oil to lecithin of 1:1 contained 10 mg/mL of soybean oil and 10 mg/mL of lecithin. Furthermore, 2-AP

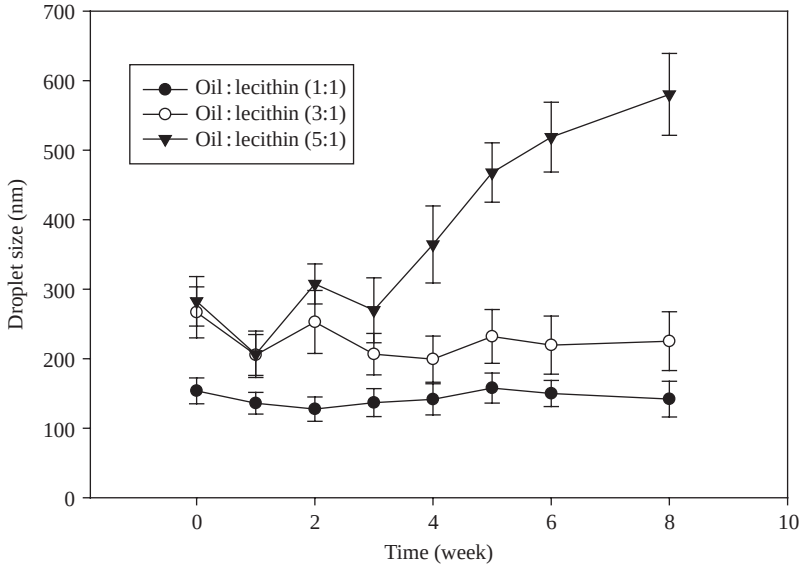

FIGURE 2. Effect of ratio of soybean oil to soybean lecithin on the changes of droplet sizes of lipid emulsions. The lipid emulsions with the ratio of 1:1 contained 1% 2-AP, 1% soybean oil, and 1% lecithin. The lipid emulsions with the ratio of 3:1 contained 1% 2-AP, 3% soybean oil, and 1% lecithin. The lipid emulsions with the ratio of 5:1 contained 1% 2-AP, 5% soybean oil, and 1% lecithin. Each value represents the mean ± *SD* ($n = 3$).

(10 mg/mL)-loaded lipid emulsions with the ratio of soybean oil to lecithin of 5:1 contained 50 mg/mL soybean oil and 10 mg/mL lecithin. Generally, as the drug content increased, the excessive drug was not incorporated within the core of lipid emulsion and was attached to the lipid membrane; thus, the droplet size of lipid emulsion increased (Gursoy & Benita, 2004). Furthermore, the droplet size of particulate delivery systems had an effect on the behavior of the drug in the body (Floyd, 1999; Liu, Mori, & Huang, 1992). Because soybean oil hardly affected the droplet size of lipid emulsion at the dose of 10 mg/mL 2-AP in the preliminary study (data not shown), all lipid emulsions were prepared with the dose of 10 mg/mL of 2-AP. The higher the ratio of oil to lecithin, the larger the droplet size of lipid emulsion (Figure 2). The droplet sizes of the lipid emulsions with the ratio of oil to lecithin of 3:1 and 5:1 increased with time. However, the lipid emulsions with the ratio of oil to lecithin of 1:1 gave no significant change in droplet size for 8 weeks, indicating that it was physically stable (Constantinides & Yiv, 1995; Laval-Jeantet, Laval-Jeantet, & Bergot, 1982). Consequently, the ratio of oil to lecithin of 1:1 was selected due to its smallest droplet size and good stability.

To investgate whether the ratio of carrier to drug affected the changes of droplet sizes of lipid emulsions, 2-AP (10 mg/mL)-loaded lipid emulsions with the ratio of carrier to drug of 2:1, 4:1, 6:1, 8:1, and 10:1 were prepared, respectively, and their droplet sizes were investigated (Figure 3). The carrier was composed of the same amount of oil and lecithin. As the ratio of carrier to drug was increased to the ratio of 8:1, the droplet size of lipid emulsion was decreased. Thus, the ratio carrier to drug of 8:1 was selected to prepare 2-AP-loaded lipid emulsion.

To investgate whether the stabilizer which was used often in lipid emulsion formulation affects the droplet size of lipid emulsion, 1% stabilizer such as cholesterol, linoleic acid, oleic acid, poloxamer 188, and HCO60 was added to 2-AP (10 mg/mL)-loaded lipid, respectively, and their droplet sizes were measured for 8 weeks (Figure 4).

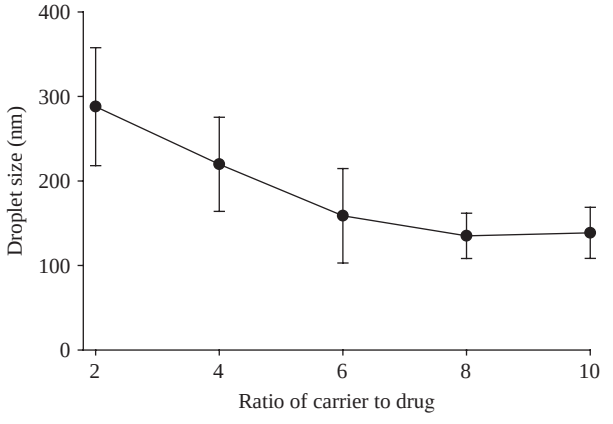

FIGURE 3. Effect of ratio of carrier to drug on the changes of droplet sizes of lipid emulsions. The ratio of carrier to drug means the ratio of the total mixture of the same weight of soybean oil and lecithin against 2-AP. Each value represents the mean ± *SD* (n = 3).

The lipid emulsions with cholesterol, linoleic acid, oleic acid, and poloxamer gave relatively larger droplet size and increased the droplet size with time. However, the droplet size of lipid emulsions with HCO60 and the droplet size without stabilizer were relatively small and hardly changed with time. However, HCO60 could not be used in parenteral formulation due to its hemolysis property (Sha et al., 2005). No addition of stabilizer was decided, because the lipid emulsion without stabilizer was physically stable for 8 weeks. Thus, a 2-AP-loaded lipid emulsion composed of 1% 2-AP, 4% soybean oil, 4% lecithin, and 91% water was selected as a suitable formulation. Furthermore, this 2-AP-loaded lipid emulsion was isotonic with the osmotic pressure of 295 ± 17 mOsm.

The chemical stability of 2-AP in this lipid emulsion was evaluated by drug contents. The drug contents in the lipid emulsion were above 95% for 8 weeks and was not significantly changed, indicating that it was chemically stable at least for 8 weeks.

Figure 5 shows the profiles of mean plasma concentration of 2-AP after intravenous administration of lipid emulsion and PEG solution in rats with the dose of 50 mg/kg. The lipid emulsion contained 10 mg/mL 2-AP, 4 mg/mL soybean oil, and 4 mg/mL lecithin. The lipid emulsion gave lower plasma concentrations of 2-AP than did 2-AP solution. In particular, the plasma concentrations of 2-AP in lipid emulsion, from 2 to 6 h, were significantly higher compared with those in 2-AP solution ($p < .05$). Our results suggested that 2-AP formulated with lipid emulsion was distributed to organs more than PEG solution.

The pharmacokinetic parameters of 2-AP were determined after intravenous administration of PEG solution and lipid emulsion (Table 1). The half life, AUC, and MRT of 2-AP in the lipid emulsion were lower than those of 2-AP solution. Furthermore, 2-AP in lipid emulsion was significantly cleared and distributed in organs. These results suggest that 2-AP in lipid emulsion was faster cleared compared with 2-AP solution, because the lipid emulsion might be captured by reticuloendothelial system (RES) in the liver (Liu et al., 1992).

The tissue distribution of 2-AP were determined 1 h after intravenous administration of solution and lipid emulsion (Figure 6). The lipid emulsion showed significantly higher *T*/*P* ratio in liver, kidney, lung, and spleen compared with PEG solution ($p < .05$). In this study, about 6.35% and 3.91% of drug administered was obtained for the lipid emulsion and drug solution, respectively. The amount of 2-AP obtained in lipid emulsion administration from each tissue were 4.73 ± 0.83% in liver, 0.21 ± 0.03% in heart, 0.96 ± 0.16% in kidney, 0.23 ± 0.04% in lung, and 0.21 ± 0.03% in spleen whereas in PEG solution, 3.12 ± 0.55%, 0.14 ± 0.02%, 0.51 ± 0.08%, 0.1 ± 0.01%, and 0.05 ± 0.01%, respectively.

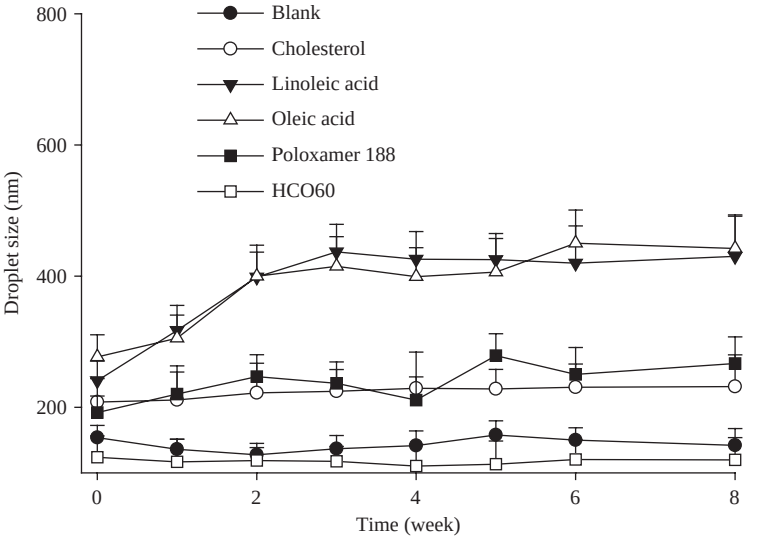

FIGURE 4. Effect of stabilizer on the changes of droplet size of lipid emulsion. Each stabilizer was added by 1% (wt/vol). One percent of stabilizer such as cholesterol, linoleic acid, oleic acid, poloxamer 188, and HCO60 were added to 2-AP (10 mg/mL)-loaded lipid emulsions, respectively. 2-AP (10 mg/mL)-loaded lipid emulsions contained 10 mg/mL 2-AP, 4 mg/mL soybean oil, and 4 mg/mL lecithin. Each value represents the mean ± *SD* (n = 3).

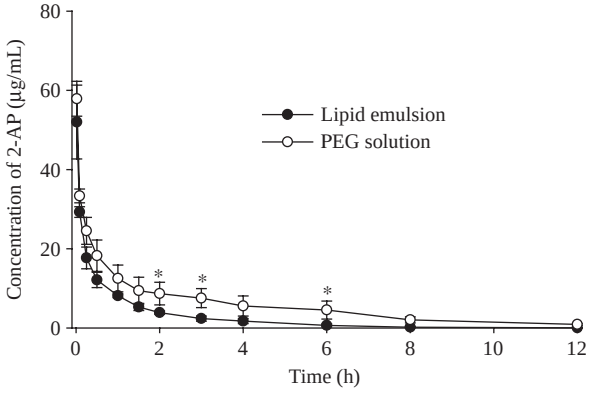

FIGURE 5. Plasma concentration-time profiles of 2-AP after 1-min intravenous infusion of drug and lipid emulsion (50 mg/kg). 2-AP (10 mg/mL)-loaded lipid emulsions contained 10 mg/mL 2-AP, 4 mg/mL soybean oil, and 4 mg/mL lecithin. Each value represents the mean ± *SD* (n = 6). *p < .05 compared with PEG solution.

Furthermore, among the organs tested, *T*/*P* ratio in the liver was highest. This result means that 2-AP in the lipid emulsion gave a higher affinity to the organs, especially the liver, compared with 2-AP in solution (Kim et al., 1997). This high affinity could be contributed not only by a characteristic of particulate delivery system that is easily captured by RES in liver, but also by an allylsulfur moiety of 2-AP (Kang et al., 2001; Lee et al., 2004).

From these findings, 2-AP could be selectively delivered to the liver, because it was speedily cleared in blood and distributed in organs, especially the liver. Thus, it might be used as a chemoprotective agent with an effective action for the liver enzyme.

TABLE 1
Pharmacokinetic Parameters of 2-AP after 1-min Intravenous Infusion of PEG Solution and Lipid Emulsion to Rats (50 mg/kg)

Parameters	PEG Solution	Lipid Emulsion
$t_{1/2}$ (h)	2.89 ± 0.79	1.46 ± 0.20*
AUC (μg h/mL)	71.15 ± 17.28	29.53 ± 2.85*
MRT (h)	3.85 ± 0.71	1.59 ± 0.19*
CL (mL/h kg)	875.19 ± 198.57	2,044.48 ± 194.69*
V_{dss} (mL/kg)	3,378.51 ± 968.69	3,242.41 ± 252.57*

Each value represents the mean ± *SD* (n = 6).
*p < .05 compared to PEG solution.

CONCLUSION

The lipid emulsion composed of 1% 2-AP, 4% soybean oil, 4% lecithin, and 91% water was physically and chemically stable for 8 weeks. It gave significantly faster clearance of 2-AP and higher affinity to the organs, especially the liver, compared with the drug solution. Thus, the lipid emulsion formulated with soybean oil and lecithin could be used as a potential dosage form with the liver targeting and enhanced stability of sparingly water-soluble 2-AP.

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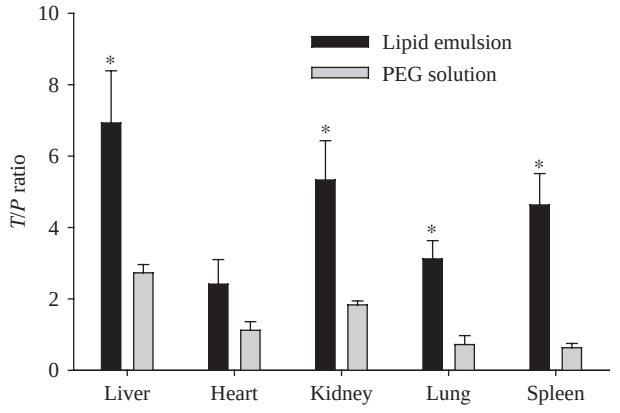

FIGURE 6. Tissue distribution of 2-AP after intravenous administration of drug and lipid emulsion 1 h after 1-min intravenous infusion (50 mg/kg). 2-AP (10 mg/mL)-loaded lipid emulsions contained 10 mg/mL 2-AP, 4 mg/mL soybean oil, and 4 mg/mL lecithin. Each value represents the mean ± *SD* (n = 6). *$p < .05$ compared with PEG solution.

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