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Pharmacokinetic study of the structural components of adenosine diphosphate-encapsulated liposomes coated with fibrinogen γ-chain dodecapeptide as a synthetic platelet substitute

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Running title: Disposition of H12-(ADP)-liposomes

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Abbreviation: RGD, arginine-glycine-aspartic acid; **ADP**, adenosine diphosphate; **H12**, HHLGGAKQAGDV; **H12-(ADP)-liposome**, ADP-encapsulated liposomes modified with a dodecapeptide; **GP**, glycoprotein; **PEG**, polyethyleneglycol; **DPPC**, 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine; **PEG-DSPE**, 2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-N-[monomethoxypoly(ethylenegly

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col)]; **DHSG**, 1,5-Dihexadecyl-*N*-succinyl-L-glutamate; **HPLC**, high performance liquid chromatography; **ID**, injected dose; **HbV**, hemoglobin-vesicles; **MPS**, mononuclear phagocyte system.

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Abstract

Fibrinogen γ-chain (dodecapeptide HHLGGAKQAGDV, H12)-coated, adenosine-diphosphate (ADP)-encapsulated liposomes (H12-(ADP)-liposomes) were developed as a synthetic platelet alternative that specifically accumulates at bleeding sites as the result of interactions with activated platelets via GPIIb/IIIa and augments platelet aggregation by releasing ADP. The aim of this study is to characterize the pharmacokinetic properties of H12-(ADP)-liposomes and structural components in rats, and to predict the blood retention of H12-(ADP)-liposomes in humans. With use of H12-(ADP)-liposomes in which the encapsulated ADP and liposomal membrane cholesterol were radiolabled with ^{14}C and ^{3}H, respectively, it was found that the time courses for the plasma concentration curves of ^{14}C and ^{3}H radioactivity showed that the H12-(ADP)-liposomes remained intact in the blood circulation for up to 24 h after injection, and were mainly distributed to the liver and spleen. However, the ^{14}C and ^{3}H radioactivity of H12-(ADP)-liposomes disappeared from organs within 7 day after injection. The encapsulated ADP was metabolized to allantoin, which is the final metabolite of ADP in rodents, and was mainly eliminated in the urine, while the cholesterol were mainly eliminated in feces. In addition, the half-life of the H12-(ADP)-liposomes in humans was predicted to be approximately 96 hrs from pharmacokinetic data obtained for mice, rats and rabbits using an allometric equation. These results suggest that H12-(ADP)-liposome has potential with proper pharmacokinetic and acceptable biodegradable properties as synthetic platelet substitute.

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Introduction

As the numbers of patients with hematologic malignancies and solid tumors increase, platelet transfusion represents one of the most essential prophylactic or therapeutic treatments, because these disorders induce severe thrombocytopenia caused by the intensive chemotherapy, surgical procedures and radiotherapy. However, platelet transfusion can introduce a variety of complications such as bacterial infection, allergic reaction and acute lung injury. In addition, donated platelet for blood transfusions can only be stored for a period of 4 days in Japan and 5-7 days in the USA and Europe. This has become a serious concern in our aging society and a stable supply in an emergency situation such as disasters and pandemics needs to be on hand. To solve these problems, various platelet substitutes, which consist of materials derived from blood components, have been developed (Blajchman, 2003), such as solubilized platelet membrane protein conjugated liposomes (Plateletsome) (Rybak and Renzulli, 1993), infusible platelet membranes (IPM) (Graham et al., 2001), fibrinogen-coated albumin microcapsules (Synthocyte) (Levi et al., 1999), red blood cells with bound fibrinogen (Agam and Livne, 1992), liposomes bearing fibrinogen (Casals et al., 2003), arginine-glycine-aspartic acid (RGD) peptidebound red blood cells (Thromboerythrocyte) (Coller et al., 1992) and fibrinogen-conjugated albumin polymers (Takeoka et al., 2001). However, these platelet substitutes have not yet been approved for clinical use.

Adenosine diphosphate (ADP)-encapsulated liposomes modified with a dodecapeptide (HHLGGAKQAGDV, H12) (H12-(ADP)-liposome) was developed as a new type of synthetic platelet alternative. The glycoprotein (GP) IIb/IIIa, which is present on the platelet membranes, is converted from an inactive to an active form when

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platelets adhere to collagen that is exposed on sites of vascular injury (Takagi et al., 2002; Xiao et al., 2004), and platelet aggregation is mediated by fibrinogen by bridging adjacent platelets through GPIIb/IIIa in an activation-dependent manner in the circulation. Among several GPIIb/IIIa recognized sequence sites in fibrinogen such as the RGD-based sequences (^{95}RGDF^{98} and ^{572}RGDS^{575} in the Aα chains) and H12 (^{400}HHLGGAKQAGDV^{411}) in the carboxy-terminus of the γ-chain (Kloczewiak et al., 1982; Kloczewiak et al., 1984; Hawiger et al., 1989), H12 is a specific binding site of the ligand for activated GPIIb/IIIa (Lam et al., 1987; Andrieux et al., 1989), whereas RGD related peptides are non-specific with respect to a wide variety of integrins from various cell types (Phillips et al., 1991). In addition, when ADP is released from activated platelets, it functions as potent platelet agonist. Thus, these modifications to H12-(ADP)-liposomes enable them to specifically interact with activated platelets, resulting in platelet aggregation. In fact, H12-liposomes with polyethyleneglycol (PEG)-surface modification specifically accumulate at the site of an injury in vivo and were determined to shorten bleeding time in a dose-dependent manner in a thrombocytopenic rat and a rabbit model (Okamura et al., 2005; Okamura et al., 2009; Okamura et al., 2010a; Okamura et al., 2010b; Nishikawa et al., 2012). Therefore, these findings prompted us to conclude that H12-(ADP)-liposomes have considerable potential for use as an alternative for actual platelets in clinical settings.

Before new drugs are approved for clinical use, they are required to undergo a wide variety of evaluations, including physicochemical tests, pre-clinical studies and clinical trials. As described above, pre-clinical studies of H12-(ADP)-liposomes have resulted in pharmacological evidence to indicate that they can be used as a platelet substitute (Okamura et al., 2005; Okamura et al., 2009; Okamura et al., 2010a; Okamura

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et al., 2010b; Nishikawa et al., 2012). However, information concerning pharmacokinetic properties is lacking, especially the disposition and retention of each component in tissues after injection. Our strategy for the development of H12-(ADP)-liposome is based on the fact that, not only better pharmacological effects, but also acceptable biodegradable properties (no accumulation or retention) need to be documented. In addition, pre-clinical pharmacokinetic studies in various mammalian species are essential, as the results of such studies can be extrapolated to humans, allowing appropriate dosing regimens to be estimated in the case of humans.

In the present study, we report on an evaluation of the pharmacokinetic properties of the H12-(ADP)-liposomes and components thereof, from the standpoint of stability in the blood circulation and the metabolism and excretion of each component. For this purpose, we prepared H12-(ADP)-liposomes that were ^{14}C, ^{3}H double radiolabeled, in which the encapsulated ADP and membrane component (cholesterol) were labeled with ^{14}C and ^{3}H, respectively. Furthermore, we predicted some important pharmacokinetic parameters, especially retention in the blood circulation, in humans, based on data obtained in pharmacokinetic studies in mice, rats and rabbits.

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Materials and Methods

Reagents

Cholesterol and 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) were purchased from Nippon Fine Chemical (Osaka, Japan), and 2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-N-[monomethoxypoly(ethyleneglycol)] (PEG-DSPE, 5.1 kDa) was from NOF (Tokyo, Japan). 1,5-Dihexadecyl-*N*-succinyl-L-glutamate (DHSG) and H12-PEG-Glu2C18, in which the fibrinogen γ-chain dodecapeptide (C-HHLGGAKQAGDV, Cys-H12) was conjugated to the end of the PEG-lipids, were synthesized as previous reported (Okamura et al., 2005). Allantoin, uric acid, hypoxantine, xanthine and ADP were obtained from Sigma-Aldrich (St Louis, MO, USA).

Preparation of ^{14}C, ^{3}H double labled H12-(ADP)-liposomes

Firstly, ^{14}C labeled H12-(ADP)-liposomes were prepared under sterile conditions as previously reported, with minor modifications (Okamura et al., 2009). In brief, DPPC (1000 mg, 1.36 mmol), cholesterol (527 mg, 1.36 mmol), DHSG (189 mg, 272 μmol), PEG-DSPE (52 mg, 9.0 μmol) and H12-PEG-Glu2C18 (47 mg, 9.0 μmol) were dissolved in *t*-butyl alcohol and then freeze-dried. The resulting mixed lipids were hydrated with phosphate-buffered saline (pH 7.4) containing ADP (1 mM) and [8-^{14}C]ADP (1.85 MBq; Moravec Biochemicas, Inc., USA), and extruded through membrane filters (pore size, 0.22 μm; Durapore®; Millipore, Tokyo, Japan). Liposomes were washed with phosphate-buffered saline by centrifugation (100000 g, 30 min, 4°C), and the remaining ADP was eliminated by sephadexG25. The diameter and Zeta-potential of the ^{14}C labeled H12-(ADP)-liposomes used in this study are regulated

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at 250 ± 50 nm and -10 ± 0.9 mV, respectively. The 5-10% of added ADP was encapsulated in the inner space of the vesicle.

The ^{3}H labeling of ^{14}C labeled H12-(ADP)-liposomes, to prepare ^{14}C and ^{3}H double labeled H12-(ADP)-liposomes was carried out according to a previous report (Taguchi et al., 2009). The ^{14}C labeled H12-(ADP)-liposomes (1 mL) was mixed with [1,2-^{3}H(N)]-cholesterol solution (10 μL), (PerkinElmer, Yokohama, Japan) and incubated for 12 hrs at room temperature. ^{14}C, ^{3}H labeled H12-(ADP)-liposomes were filtered through a sterile filter to remove aggregates (pore size, 450 nm). Before being used in pharmacokinetic experiments, all of the samples were mixed with unlabeled H12-(ADP)-liposomes. To employ the same procedure using H12-(ADP)-liposomes and [1,2-^{3}H(N)]-cholesterol, ^{3}H labeled H12-(ADP)-liposomes, which did not contain [8-^{14}C]ADP, were prepared for the pharmacokinetic studies in mice and rabbits.

Animals

All animal experiments were undertaken in accordance with the guideline principle and procedure of Kumamoto University for the care and use of laboratory animals. Experiments were carried out with male ddY mice (28-30 g body weight; Japan SLC, Inc. Shizuoka Japan), male Sprague-Dawley (SD) rats (180-210 g body weight; Kyudou Co. Kumamoto, Japan) and male New Zealand White (NZW) rabbits (2.0-2.2 kg body weight; Biotek Co. Saga, Japan). All animals were maintained under conventional housing conditions, with food and water *ad libitum* in a temperature-controlled room with a 12-hrs dark/light cycle.

Pharmacokinetic studies

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Administration and collecting blood and organs in rats

Twenty-four SD rats were anesthetized with diethyl ether and received a single injection of ^{14}C, ^{3}H labeled H12-(ADP)-liposomes (10 mg lipids/kg (n=16), 20 mg lipids/kg (n=4) and 40 mg lipids/kg (n=4)). In all rat groups, four rats were selected to undergo the plasma concentration test. Under ether anesthesia, approximately 200 μL blood samples in all administration groups were collected from tail vein at multiple time points after the injection of the ^{14}C, ^{3}H labeled H12-(ADP)-liposomes (3, 10, 30 min, 1, 2, 3, 6, 12, 24, 48 and 168 hrs) and the plasma was separated by centrifugation (3000 g, 5 min). After collecting the last blood sample (168 hrs), the rats were sacrificed for excision of organs (kidney, liver, spleen, lung and heart). Urine and feces were collected at fixed intervals in a metabolic cage. In addition, the four rats were sacrificed and organs were collected at 2, 6, 24 hrs after an injection of ^{14}C, ^{3}H labeled H12-(ADP)-liposomes at a dose of 10 mg lipids/kg.

Administration and collection of blood and organs in mice and rabbits

Twenty-eight ddY mice received a single injection of ^{3}H labeled H12-(ADP)-liposomes (10 mg lipids/kg) in the tail vein under ether anesthesia. At each time after the injection of ^{3}H labeled H12-(ADP)-liposomes (3, 30 min, 1, 3, 6, 12, 24 hrs), four mice were anesthetized with ether and blood was collected from the inferior vena cava, and plasma was obtained by centrifugation (3000 g, 5 min).

Four NZW rabbits received a single injection of ^{3}H labeled H12-(ADP)-liposomes at a dose of 10 mg lipids/kg. The blood was collected from the auricular veins at each time after injection (3, 10, 30 min, 1, 2, 12, 24, 36, 48, 72 hrs), and plasma was obtained by centrifugation (3000 g, 5 min).

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Measurement of ^{14}C and ^{3}H radioactivity

Plasma samples were solubilized in a mixture of Soluene-350 (Perkin Elmer, Yokohama, Japan) and isopropyl alcohol (at a ratio of 1/1) for 24 hrs at 50°C. The organ samples were rinsed with saline, minced, and solubilized in Soluene-350 for 24 hrs at 50°C. Urine and feces were also weighed and solubilized in Soluene-350. All samples were decolorized by treatment with a hydrogen peroxide solution after treatment of Soluene-350 or isopropyl alcohol. The ^{14}C, ^{3}H radioactivity was determined by liquid scintillation counting (LSC-5121, Aloka, Tokyo, Japan) with Hionic Fluor (Perkin Elmer, Yokohama, Japan).

Analysis of metabolites of encapsulated ADP

ADP metabolites in urine were determined by high performance liquid chromatography (HPLC), as described previously (George et al., 2006). A part of the urine obtained in the pharmacokinetic study in rats was used for this analysis, and aliquots of urine samples (2.5 mL) were mixed with 200 μL of 10% sulphuric acid. Just before the analysis, the urine samples were centrifuged and filtered through a Dismic-25cs (ADVANTEC, Tokyo, Japan, 0.2 、μm pore size) and diluted ten-fold with water after adjusting the pH to 7 with 0.01N sodium hydroxide and 0.01N sulphuric acid. A standard solution containing ADP, allantoin, uric acid, hypoxantine and xanthine was prepared as reported in a previous study (George et al., 2006). The HPLC system consisted of a Waters 2695 pump (Waters, Massachusetts, USA), a Waters 2487 detector (Waters, Massachusetts, USA) operated at 220 nm. LC analyses were achieved with a 250 × 4 mm, 5 μm LiChrospher® 100 RP-18 endcapped column (LiChroCART®

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250-4, Merck, Darmstadt, Germany). Furthermore, each ADP metabolite separated by HPLC was collected by a fraction collector (CHF121SA, ADVANTEC, Tokyo, Japan) and ^{14}C radioactivity was determined by liquid scintillation counting with Hionic Fluor.

Interspecies scaling of pharmacokinetic parameters

Allometric relationships between various pharmacokinetic parameters (P) and body weight (W) were plotted on a log-log scale. Linear regression of the logarithmic values was calculated using the least-squares method using Eq. (1) (Boxenbaum, 1984).

$$P = \alpha \cdot W^{\beta} \qquad (1)$$

P is the parameter of interest (distribution volume (V_{dss}) or clearance (CL)), W is the body weight (kg), and α and β are the coefficient and exponent of the allometric equation, respectively. The average body weights of 0.034 kg (mouse), 0.242 kg (rat), 2.08 kg (rabbit) and 70 kg (human) were used for prediction of V_{dss} and CL for human. After predicting of V_{dss} and CL for humans (70 kg) using Eq. (1), the half-life for human was estimated.

Data Analysis

A non-compartment model was used for the pharmacokinetic analysis. Each parameter, half-life ($t_{1/2,}$ hr), mean residence time (MRT, hr), area under the concentration-time curve (AUC, hr・% of dose/mL), clearance (CL, mL/hr), distribution volume (V_{dss}, mL), was calculated using the moment analysis program available on Microsoft Excel. (Yamakawa et al., 2013) Data are shown as means ± SD for the indicated number of animals.

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Results

Pharmacokinetics of H12-(ADP)-liposome components in rats

In order to investigate the pharmacokinetics of each component of the H12-(ADP)-liposomes, ^{14}C, ^{3}H labeled H12-(ADP)-liposomes, in which the encapsulated ADP was labeled with ^{14}C and the membrane component (cholesterol) was labeled with ^{3}H, were prepared (Figure 1A). As shown in Fig. 1B and Table 1, the plasma concentration curves and pharmacokinetic parameters for ^{14}C radioactivity and ^{3}H radioactivity were similar. These data indicate that the structure of the H12-(ADP)-liposomes remained intact in the blood circulation for periods of up to 24 hrs after injection in rats.

Moreover, we evaluated the tissue distribution of both the encapsulated ADP and membrane component (cholesterol) of the H12-(ADP)-liposomes. Figure 2 shows the tissue distribution in organs at 2, 6 and 24 hrs after the administration of ^{14}C, ^{3}H labeled H12-(ADP)-liposomes at a dose of 10 mg lipids/kg to rats. Among these organs, the majority of both the ^{14}C and ^{3}H radioactivity of the H12-(ADP)-liposomes were distributed in the liver and spleen. However, both the ^{14}C and ^{3}H radioactivity of the H12-(ADP)-liposomes were eliminated from each organ, and the activity essentially disappeared within 7 days after injection (data not shown). These data indicate that the H12-(ADP)-liposomes are mainly distributed to the liver and spleen, but the retention in these organs is negligible.

In order to identify the excretion pathway of the H12-(ADP)-liposomes, the levels of ^{14}C and ^{3}H in urine and feces were measured (Fig. 3A and B). The ^{14}C was excreted mainly in the urine (80.4±4.9 % of the injected dose (ID) at 7 days after injection), but was low in feces (7.6±2.7 % of ID at 7 day after injection). On the other

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hand, the majority of the ^{3}H was excreted in the feces (74.2±5.7% % of ID at 7 days after injection), and excretion into the urine was essentially nil. In addition, as shown in Figure 3C, it is well known that, in rodents, endogenous ADP is ultimately metabolized to allantoin and excreted. Thus, we qualitatively determined the fate of the encapsulated ADP of the H12-(ADP)-liposomes using an HPLC method. Figure 3D shows the separated peaks for ADP and its metabolites in the standard solution and in a urine sample 6 hours after the administration of the H12-(ADP)-liposomes to a rat. Furthermore, to exclude the effect of endogenous ADP and its metabolites, we measured the ^{14}C radioactivity of each peak that had been separated by HPLC. As a result, almost all the ^{14}C radioactivity was detected in the peak corresponding to allantoin, which is the final metabolite of ADP in rodents, in the urine sample (Table 2).

These results indicate that more than 75% of each structural component of the H12-(ADP)-liposome is excreted from the body within 7 days after injection, and the encapsulated ADP and membrane component (cholesterol) derived from H12-(ADP)-liposomes were metabolized to final metabolites and excreted into the urine and feces, respectively.

Dose-dependency of H12-(ADP)-liposomes pharmacokinetics.

Figure 4 shows the time courses for the plasma concentration for the ^{14}C, ^{3}H labeled H12-(ADP)-liposomes administered to rats at doses of 10, 20 and 40 mg lipids/kg. No significant difference was found in the plasma concentration curve or pharmacokinetic parameters among all groups (Figure 4A and B). In fact, a linear relationship between the administration dose and the area under the concentration-time curve (AUC) was found, the values for which were calculated based on the lipids

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concentration (Figure 4C). These data indicate that the disposition of the H12-(ADP)-liposomes is linear for a dose of 40 mg lipids/kg.

Moreover, the tissue distribution of both the encapsulated ADP and the membrane lipids component (cholesterol) of the ^{14}C, ^{3}H labeled H12-(ADP)-liposomes was evaluated at 7 days after the injection of H12-(ADP)-liposomes at a dose of 10, 20, 40 mg lipids/kg. The level of ^{14}C and ^{3}H radioactivity was nearly undetectable in the observed organs (kidney, liver, spleen, lung and heart) (data not shown). In addition, the radioactive ^{14}C was excreted mainly in the urine (80.4±4.9 %, 52.1±3.6 %, 58.4±7.1 % of ID at 7 days after the injection at a dose of 10, 20, 40 mg lipids/kg, respectively), but was low in feces (7.6±2.7 %, 6.5±2.9 %, 2.5±1.9 % of ID at 7 days after the injection at a dose of 10, 20, 40 mg lipids/kg, respectively). On the other hand, the majority of the radioactive ^{3}H was excreted in the feces (74.2±5.7%, 98.9±14.9 %, 70.6±6.2 % of ID at 7 days after the injection at a dose of 10, 20, 40 mg lipids/kg, respectively), and small portion of the ^{3}H radioactivity was excreted into the urine. These data indicate that more than 75% of H12-(ADP)-liposomes are eliminated within 7 days after injection and retention in the body can be limited to detect at a dose of up to 40 mg lipids/kg.

Pharmacokinetics of the H12-(ADP)-liposomes in mice and rabbits

To calculate the pharmacokinetic parameters of the H12-(ADP)-liposomes in mice and rabbits, the ^{3}H labeled H12-(ADP)-liposomes were administered to mice and rabbits at a dose of 10 mg lipids/kg. According to the pharmacokinetic parameters calculated from the plasma concentration curve, the CL and V_{dss} of the ^{3}H labeled H12-(ADP)-liposomes in mice were 0.54±0.12 mL/hr and 3.81±0.35 mL, respectively, while the values in the case of rabbits were 23.5±2.8 mL/hr and 827±163 mL,

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respectively (Supplemental Table 1).

Prediction of pharmacokinetics of the H12-(ADP)-liposomes in human.

To predict the pharmacokinetics in humans, we examined the allometric relationship between V_{dss} and body weight (Fig. 5A) and CL and body weight (Fig. 5B) in mice, rats, rabbits using the results summarized in Table 1 and supplemental Table 1. As shown in Figure 5A and B, a good correlation in both relationships was observed. Furthermore, we calculated the half-life, based on extrapolation, of the H12-(ADP)-liposomes that were administered at a dose of 10 mg lipids/kg in humans to be approximately 96 hrs.

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Discussion

In the present study, the pharmacokinetic properties of H12-(ADP)-liposomes and structural components thereof, including the encapsulated ADP and membrane components (cholesterol) were characterized. The findings confirmed that the product has proper pharmacological functions and acceptable biodegradable properties (little retention). This leads to the conclusion that the H12-(ADP)-liposomes have the potential for use as a synthetic platelet substitute from the viewpoint of the pharmacokinetic properties in rodents.

We encapsulated ADP into H12 coated liposomes to strengthen the hemostatic ability of the H12 coated liposome as a platelet substitute, because this physiologically relevant platelet agonist is stored in dense granules and released upon cellular activation, then functions to reinforce or maintain platelet aggregation through corresponding platelet nucleotide receptors P2Y1 and P2Y12. Thus, the stable encapsulation of ADP in liposomes permits them to function at sites of vascular injuries. The findings herein clearly show that, for up to 24 h after injection in rats, the plasma concentration curves for ^{14}C, ^{3}H radiolabeled H12-(ADP)-liposome exhibited similar behaviors (Figure 1), indicating that the H12-(ADP)-liposomes circulate in the bloodstream without any leakage of ADP. In addition, we also realized that the non-liposomal ADP was immediately eliminated from blood (data not shown), because ADP released into blood was metabolized by leukocytes, erythrocytes and endothelial cells (Marcus et al., 2003; Heptinstall et al., 2005). This means that ADP encapsulated in the vesicle has advantages that is not only specific delivery ADP to injury site but also improvement of the blood retention of ADP. Previous in vivo hemostatic studies of H12-(ADP)-liposomes using a rat model with busulphan-induced thrombocytopenia

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(platelet counts; $1.9 \pm 0.2\times10^5\ \mu L^{-1}$) clearly showed that the tail vein bleeding times of thrombocytopenic rats after an infusion of H12-(ADP)-liposomes (10 mg lipids/kg) were significantly reduced compared to that of controls (H12-liposome (10 mg lipids/kg) and (ADP)-liposome (10 mg lipids/kg)) (Okamura et al., 2009). Furthermore, the specific accumulation of H12-(iopamidol)-liposomes at the injury site at the rat tail vein and jugular vein were identified using an explore Locus CT system (Okamura et al., 2009; Okamura et al., 2010a). These results indicate that the H12-(ADP)-liposomes circulate in the bloodstream in a stable form until reaching the site of a vascular injury, and successfully augments hematostatic effects.

Retention in the blood is also an important factor in the evaluation of the hematostatic effects of H12-(ADP)-liposomes, because if the systemic half-life of the H12-(ADP)-liposome is too short, it cannot effectively function as a platelet substitute. From the viewpoint of future clinical applications, an allometric prediction of human pharmacokinetics based on data obtained from animal studies—so called, “animal scale-up”—is important for the determination of optimal doses and intervals (Izumi et al., 1996). In fact, we successfully predicted the blood retention properties of hemoglobin-vesicles (HbV), the liposomal characteristics of which have similar characteristics in terms of liposomal structure to H12-(ADP)-liposomes. This was accomplished using an allometric equation that is generally applied in animal scale-up studies to extrapolate the half-life of pharmaceuticals in humans. In the present study, we showed that the predicted half-life of H12-(ADP)-liposomes in humans would be approximately 96 hrs (Figure 5) using the above approach. The results obtained for a single-dose pharmacokinetic study of recombinant factor VIIa (rFVIIa), which is widely used as a hemostatic agent in clinical settings, showed that its half-life was 2-3 hrs in

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patients with hemophilia (Lindley et al., 1994). These results indicate that H12-(ADP)-liposomes would be expected to adequately function as a hemostatic agent in the treatment of massive bleeding in humans.

Since H12-(ADP)-liposomes were developed as synthetic platelet substitute, it is necessary to characterize the biodegradable properties of these particles, such as the determination of their metabolism and excretion pathways. Liposomes are generally captured and degraded by mononuclear phagocyte system (MPS) in the liver and spleen, such as by Kupffer cells and splenic macrophages (Kiwada et al., 1998). As expected, more than 10% of initial dose of the H12-(ADP)-liposomes were distributed to the liver and spleen (Figure 2), which is in good agreement with a previous *in vivo* study using HbV (Sakai et al., 2001; Sakai et al., 2004). In addition, an *in vitro* finding also reported that the specific uptake and degradation of HbV were observed only in macrophage cells but not in parenchymal and endothelial cells in the liver (Taguchi et al., 2009). Furthermore, linear pharmacokinetics were found for the H12-(ADP)-liposomes within the dose of 40 mg lipids/kg (Figure 4). These results strongly suggest that the majority of the H12-(ADP)-liposomes are also scavenged and degraded by the MPS, such as by Kupffer cells or splenic macrophages, and that this process was not saturated at a doses of 40 mg lipids/kg. However, it was observed the different amount of ^{3}H and ^{14}C distribution in liver and spleen (Figure 2). This was similar to our previous finding using HbV that inner hemoglobin was rapidly eliminated from organs to urine and outer lipid component (cholesterol) was delayed to eliminate from organs to feces (Taguchi et al., 2009).Therefore, the different elimination pathway would be related to the retention in liver and spleen. Further study will be needed in this point.

The findings herein also showed that most of the ADP in

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H12-(ADP)-liposomes was mainly metabolized to allantoin and excreted into the urine within 7 days after the injection of the ^{14}C, ^{3}H labeled H12-(ADP)-liposomes (Figure 3). It is well known that uric acid is the final metabolite of purines, such as adenosine 3', 5'-phosphate, in mammals. On the other hand, the principal metabolite of exogenous cyclic nucleotides in the rat is allantoin, and not uric acid (Coulson, 1976). Furthermore, another study showed that, in rats, hepatic uricase converts most of uric acid into allantoin, a form that allows it to be excreted in the urine more readily (Friedman and Byers, 1947). Taken together these findings indicate that the ADP encapsulated by H12-(ADP)-liposome was completely metabolized and excreted into the urine even though ADP was encapsulated within liposome. However, ^{14}C radioactivity were not completely recovered until 7 days after ^{14}C labeled H12-(ADP)-liposome administration. Although we could not explain the reason why the recovery of ^{14}C radioactivity was less than 100% at higher doses, it was suggested that a part of encapsulated ADP was used in the body as endogenous ADP.

The [^{3}H]cholesterol in H12-(ADP)-liposomes was mainly excreted into feces within 7 days after the injection of ^{3}H labeled H12-(ADP)-liposomes. This result is in good agreement with the disposition of HbV, using HbV labeled with [^{3}H] cholesterol after an injection of HbV, which revealed that the majority of outer lipids component (cholesterol) was excreted via feces within 7 days (Taguchi et al., 2009). Kuipers et al. previously reported that cholesterol in vesicles reappear in the blood mainly as lipoprotein-cholesterol complexes after entrapment in Kupffer cells and should then be excreted in the bile after entrapment of the lipoprotein cholesterol complex by hepatocytes (Kuipers et al., 1986). Therefore, a knowledge of whether the behavior of cholesterol as the lipids components of H12-(ADP)-liposome is the same as that of

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endogenous cholesterol after the metabolization of H12-(ADP)-liposome in the MPS would be highly desirable. On the other hand, we did not directly examine the disposition of the DPPC, DHSG, PEG-DSPE, and H12-PEG-Glu2C18 in H12-(ADP)-liposomes. Previous reports have shown that the phospholipids in liposome are metabolized in the MPS and reused as cell membranes or are excreted into the bile (Dijkstra et al., 1985; Verkade et al., 1991). Therefore, it is also possible that phospholipids in H12-(ADP)-liposome are also metabolized and excreted in the same manner as the other liposome components, as mentioned above.

From the standpoint of biodegradable properties, it is also important to realize the possibility that H12-(ADP)-liposomes and components might accumulate in tissues, because it is well known that cholesterol is a risk factor for several diseases, including arteriosclerosis and hyperlipidemia. The findings reported herein indicate that both H12-(ADP)-liposomes and components derived from them disappeared from the bloodstream and organs within the 7 days after the injection of the H12-(ADP)-liposomes, indicating that H12-(ADP)-liposomes and components derived from them possess low accumulative properties. Therefore, H12-(ADP)-liposomes contain the appropriate components and have the potential for use as a synthetic platelet substitute, because they possess acceptable biodegradable properties.

Based on the present findings, we provide the first demonstration to show that the disposition of H12-(ADP)-liposomes and components derived from them, occurs as follows. After being systemically administrated, the H12-(ADP)-liposomes are stable and circulate in an intact form in the circulation. As a result, some of the H12-(ADP)-liposomes would be specifically recruited at an injury site and would exert a pharmacological action, while the rest mainly are distributed to the liver and spleen,

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where they are degraded by the MPS. Finally, the encapsulated ADP and membrane components are eliminated mainly to the urine and feces, respectively, as final metabolites. In addition, our pharmacokinetic study, using different animal species, enabled us to predict that the half-life of H12-(ADP)-liposomes in humans is 96 hours. The above findings provide usable information for the development of the H12-(ADP)-liposomes for use as a platelet substitute.

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Authorship Contributions

Participated in research design: Taguchi, Otagiri and Maruyama

Conducted experiments: Taguchi, Ujihira, Ogaki, Fujiyama, and Doi

Contributed new reagents or analytic tools: Ikeda and Handa

Performed data analysis: Taguchi, Ujihira and Watanabe

Wrote or contributed to the writing of the manuscript: Taguchi, Okamura, Takeoka, Handa, Otagiri, and Maruyama

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Footnotes

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Figure legends

Figure 1

(A) Structure and regiospecifically-^{3}H, ^{14}C radiolabled of H12-(ADP)-liposome. (B) Time course for the plasma concentration of ^{3}H and ^{14}C radiolabled H12-(ADP)-liposome after intravenous injection at a dose of 10 mg lipids/kg to rats. Each point represents the mean ± S.D. (*n*=4).

Figure 2

The tissue distribution of (A) ^{3}H and (B) ^{14}C radioactivity at 2, 6, 24 hours after an intravenous injection of ^{3}H and ^{14}C radiolabled H12-(ADP)-liposome at a dose of 10 mg lipids/kg to rats. Each point represents the mean ± S.D. (*n*=4).

Figure 3

Time course for radioactivity in urine (A) and feces (B) after the administration of ^{3}H and ^{14}C radiolabled H12-(ADP)-liposome to rats. Each point represents the mean ± S.D. (*n*=4). **(C) Scheme of metabolism pathway from ADP to allantoin in rodents. (D) Chromatogram of standard mixture and urine sample analyzed by HPLC.** The standard peaks are (a) allantoin, (b) ADP, (c) uric acid, (d) hypoxanthine and (e) xanthine. The urine sample was collected 6 hour after intravenous injection of ^{3}H and ^{14}C radiolabled H12-(ADP)-liposome at a dose of 10 mg lipids/kg to rats.

Figure 4

Dose-dependent plasma concentration curve of (A) ^{3}H and (B) ^{14}C radiolabeled H12-(ADP)-liposome after intravenous injection at a dose of 10, 20 and 40 mg

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lipids/kg to rats. Each point represents the mean ± S.D. (*n*=4). **(C) Relationship between the dose of H12-(ADP)-liposome and the area under the blood concentration-time curve.** The linear regression of logarithmic values was calculated using the least-squares method (y=98.33x+124.98, r^2=1)

Figure 5

Allometric relationships between body weight and distribution volume (V_{dss}) (A) and body weight and clearance (CL) (B). The linear regression of the logarithmic values was calculated using the least-squares method (A, $y=257.71x^{1.2947}$, r^2=0.965; B, $y=10.246x^{0.8928}$, r^2=0.97). The extrapolated human values based on a body weight of 70 kg (open circle) and the values from individual animals (grey circle) are also shown.

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Table 1

The pharmacokinetic parameters of inner ADP ([8-^{14}C]ADP) and outer lipids membranes ([1,2-^{3}H(N)]-cholesterol) derived from ^{3}H and ^{14}C radiolabeled H12-(ADP)-liposomes after an intravenous injection at a dose of 10, 20 and 40 mg lipids/kg to rats.

$t_{1/2}$: half-life, MRT: mean residence time, AUC: area under the concentration-time curve, CL: clearance, V_{dss}: distribution volume

	10 mg lipid/kg		20 mg lipid/kg		40 mg lipid/kg	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
$t_{1/2}$ (hr)	8.18 ± 0.77	8.21 ± 1.01	7.48 ± 0.56	7.63 ± 0.82	6.34 ± 0.53	7.94 ± 1.10
MRT (hr)	10.2 ± 1.18	10.4 ± 1.46	9.20 ± 0.51	10.1 ± 1.00	7.65 ± 0.27	10.7 ± 0.97
AUC (hr・% of dose/mL)	58.4 ± 6.45	54.2 ± 10.1	54.0 ± 1.97	67.8 ± 3.86	56.0 ± 1.94	84.4 ± 5.68
CL (mL/hr)	1.73 ± 0.18	1.89 ± 0.32	1.85 ± 0.07	1.48 ± 0.08	1.79 ± 0.06	1.19 ± 0.07
V_{dss} (mL)	17.7 ± 3.49	19.5 ± 3.61	17.0 ± 0.58	14.8 ± 0.64	13.7 ± 0.58	12.6 ± 0.98

Each value represents the mean ± S.D. (*n*=4).

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Table 2

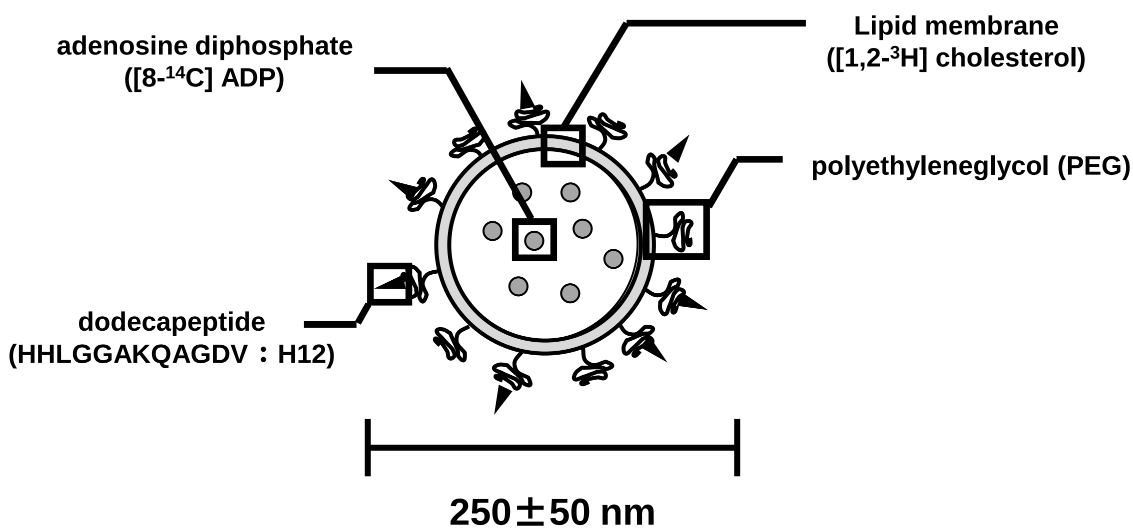
Time course for the % of total detected ^{14}C radioactivity of ADP and metabolites derived from ^{14}C-ADP in urine after intravenous injection of ^{3}H and ^{14}C radiolabled H12-(ADP)-liposome at a dose of 10 mg lipids/kg to rats.

	(a) Allantoin	(b) ADP	(c) Uric acid	(d) Hypoxanthine	(e) Xantine
6 hour	89.7±12.2	1.3±1.2	N.D.	N.D.	N.D.
1 day	78.7±13.3	16.0±15.8	N.D.	N.D.	N.D.
3 day	71.8±15.9	N.D.	N.D.	N.D.	11.2±10.5
5 day	75.0±20.8	N.D.	3.9±3.4	N.D.	6.7±5.9

N.D.; not determine, Each value represents the mean ± S.D. (*n*=4).

Figure 1

(A)


adenosine diphosphate
([8-14C] ADP)
Lipid membrane
([1,2-3H] cholesterol)
polyethyleneglycol (PEG)
dodecapeptide
(HHLGGAKQAGDV：H12)
250±50 nm

(B)

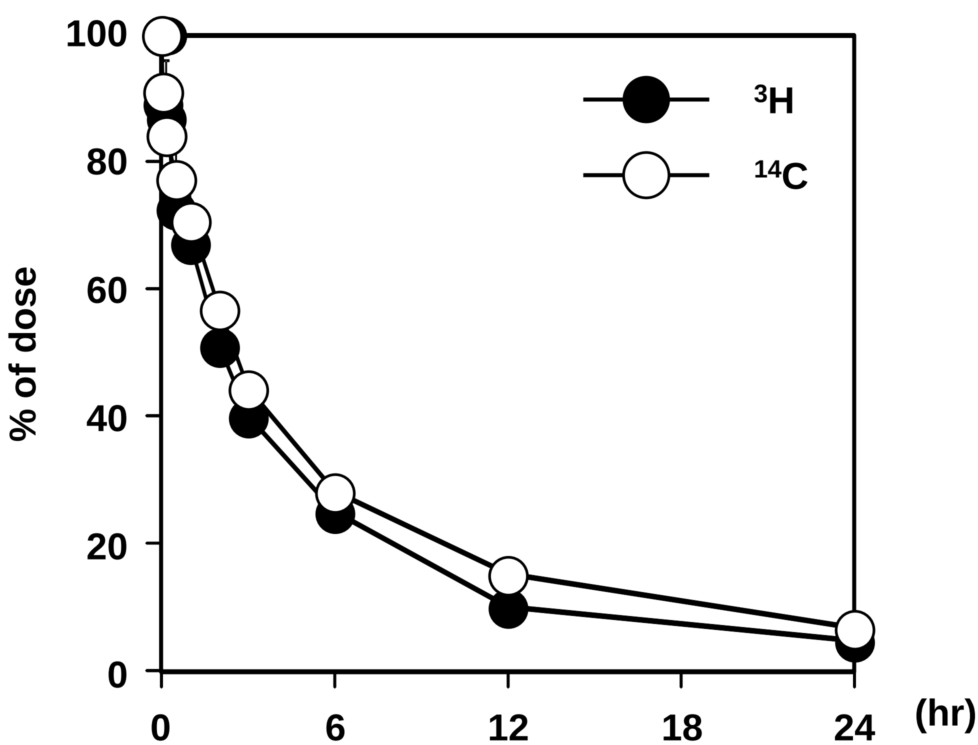
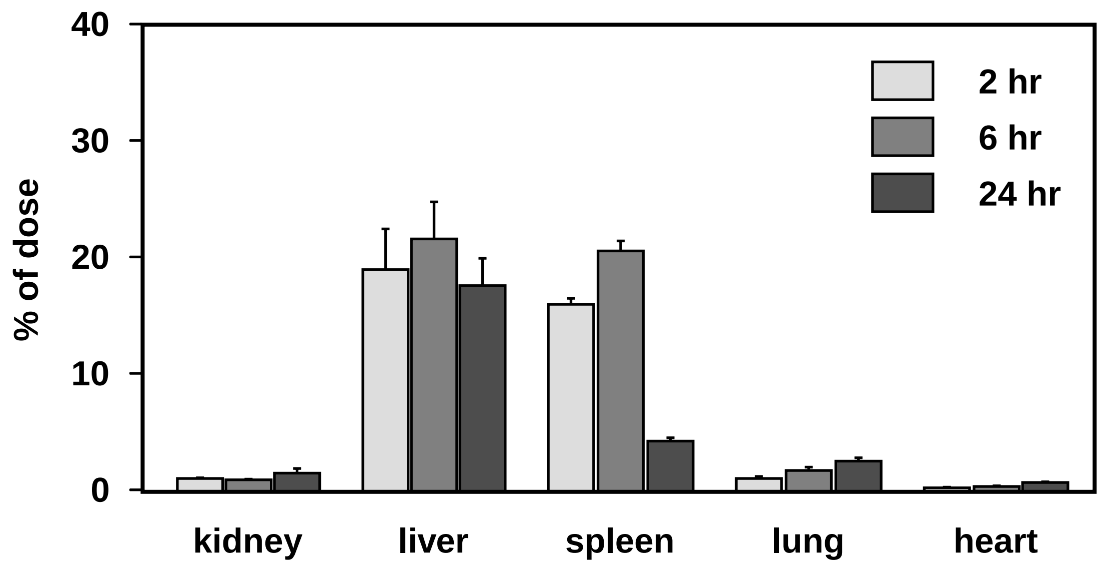

100
80
60
40
20
0
% of dose
3H
14C
0
6
12
18
24
(hr)

Figure 2

(A) ^{3}H



(B) ^{14}C

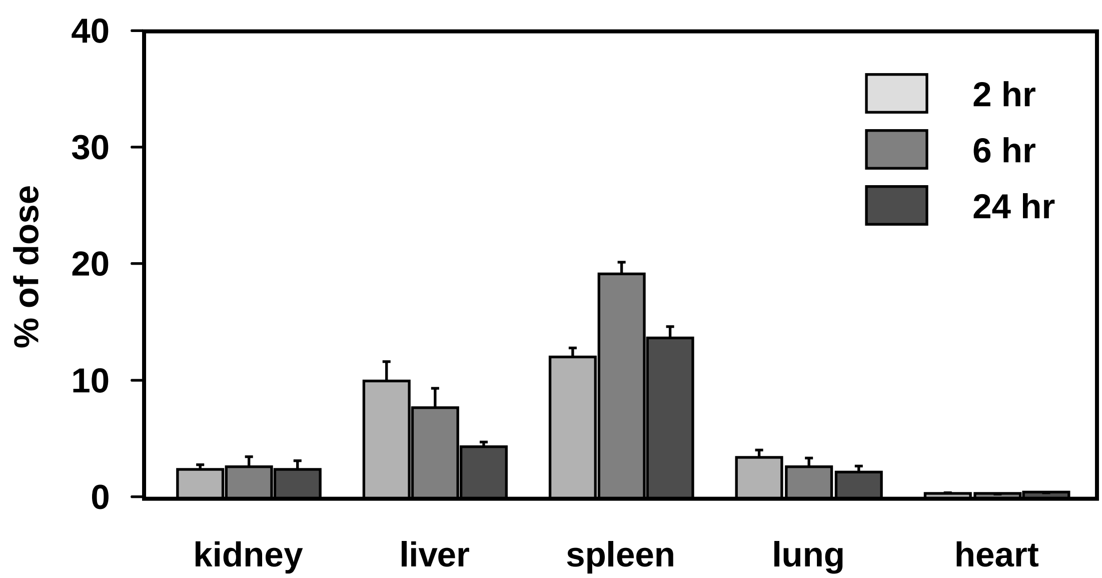


Figure 3

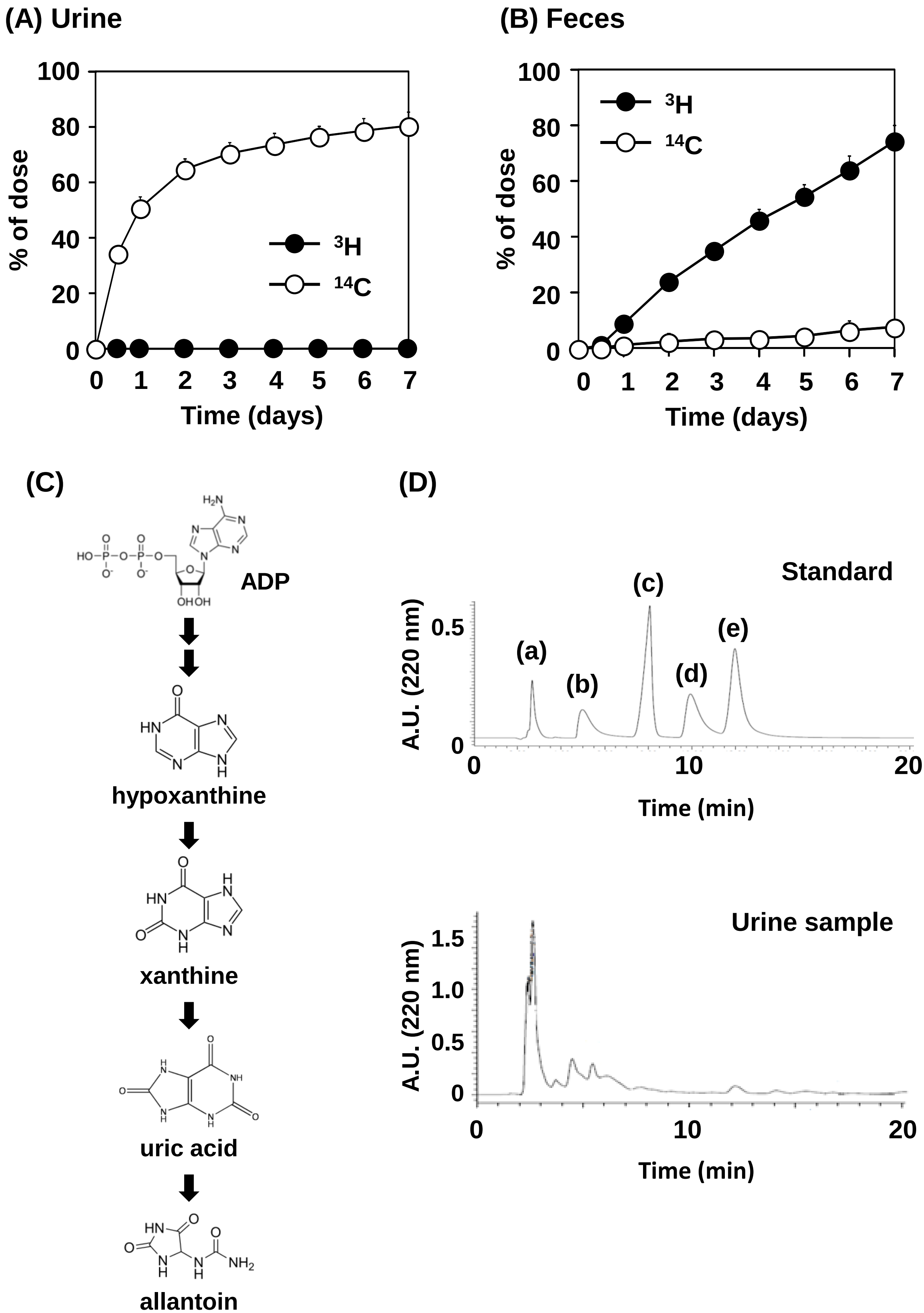
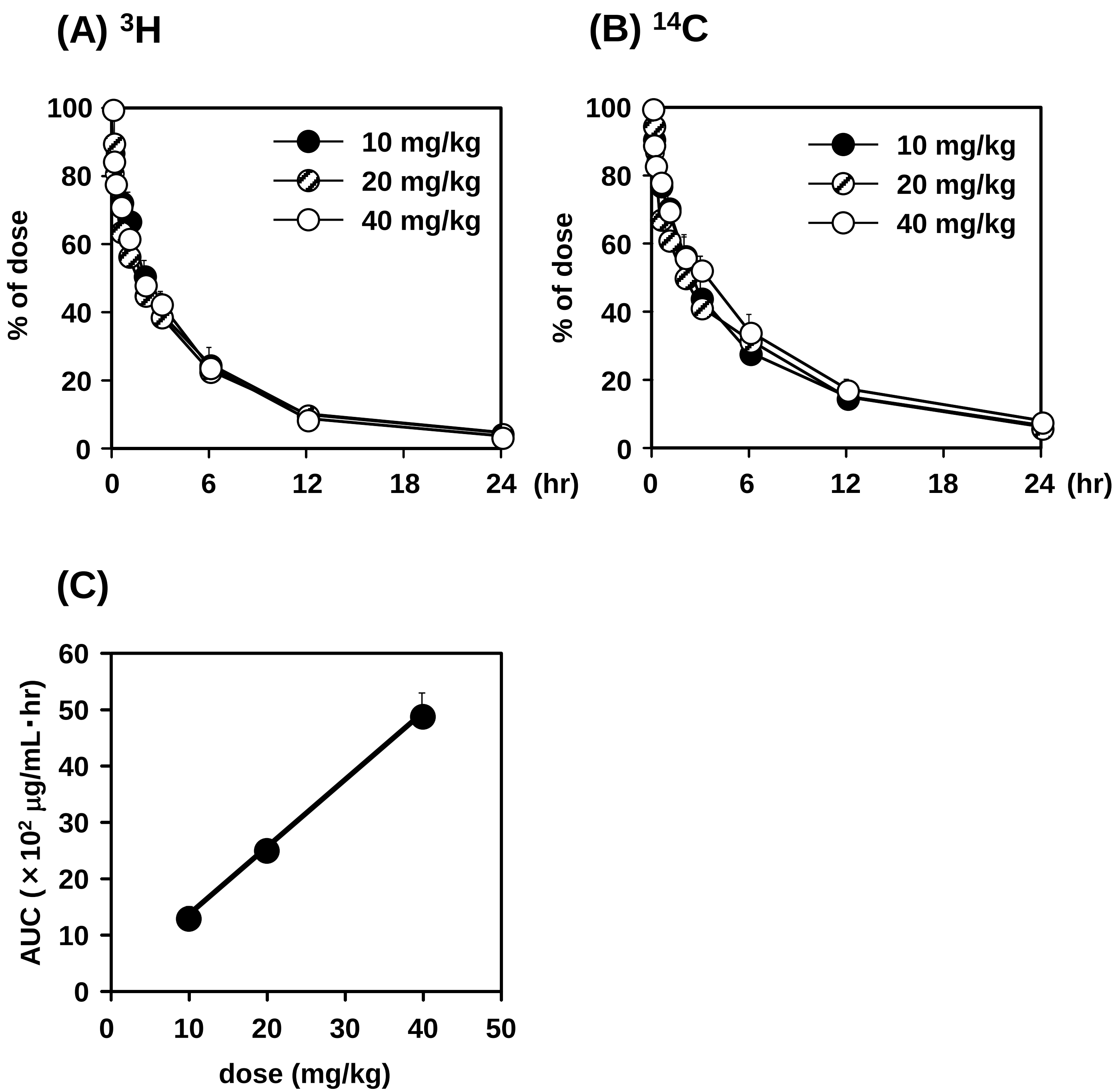

(A) Urine
(B) Feces
% of dose
Time (days)
³H
¹⁴C
0 20 40 60 80 100
0 1 2 3 4 5 6 7
(C)
ADP
hypoxanthine
xanthine
uric acid
allantoin
(D)
Standard
Urine sample
(a)
(b)
(c)
(d)
(e)
A.U. (220 nm)
Time (min)
0 10 20

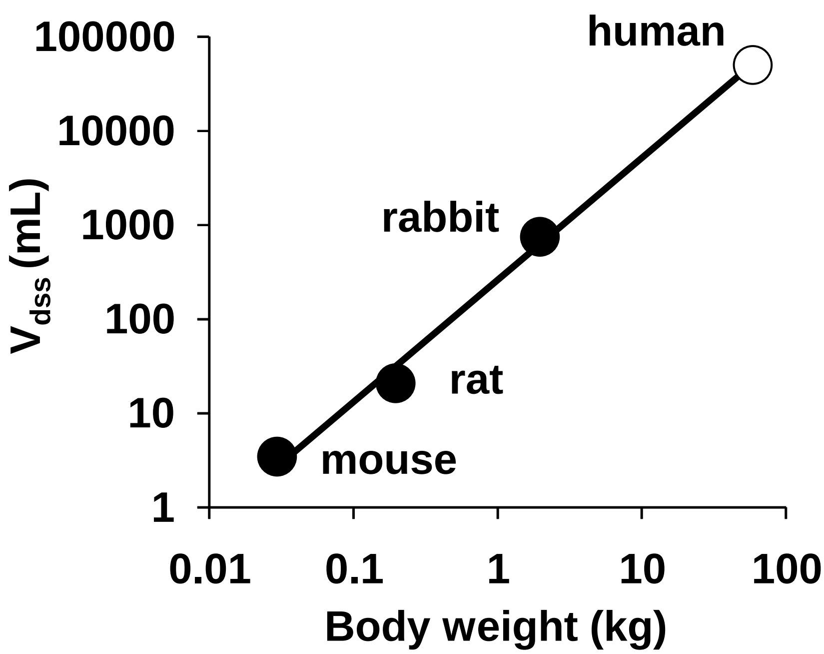
Figure 4



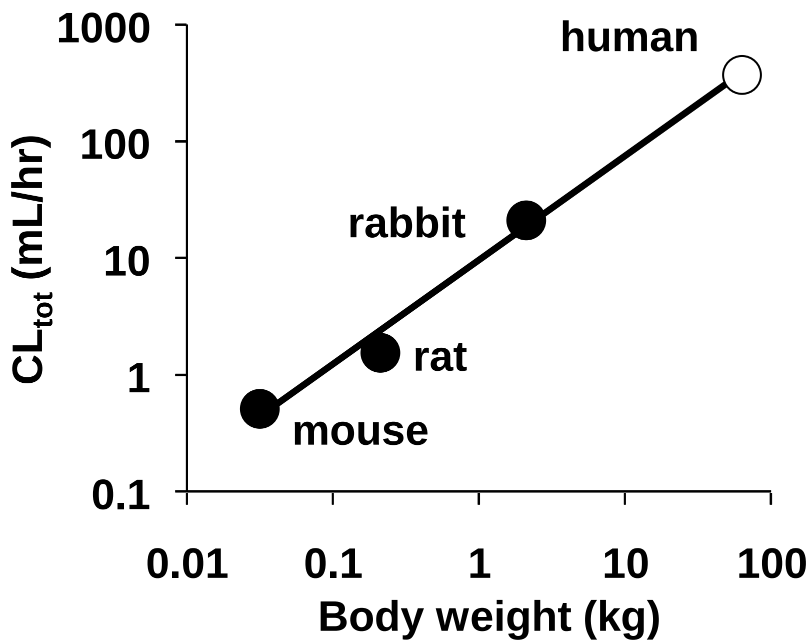
(A) ³H
(B) ¹⁴C
(C)
100
80
60
40
20
0
% of dose
0
6
12
18
24
(hr)
10 mg/kg
20 mg/kg
40 mg/kg
AUC (×10² μg/mL·hr)
dose (mg/kg)
10
20
30
40
50

Figure 5

(A)


human
rabbit
rat
mouse
100000
10000
1000
100
10
1
0.01
0.1
1
10
100
V_{dss} (mL)
Body weight (kg)

(B)


human
rabbit
rat
mouse
1000
100
10
1
0.1
0.01
0.1
1
10
100
CL_{tot} (mL/hr)
Body weight (kg)