Polysaccharides as Drug Carriers: Biodisposition of Fluorescein-Labeled Dextrans in Mice

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The biodisposition of fluorescein-labeled dextrans (FDs) with different molecular weights (MW = 4—500 kDa) was systematically examined in mice. After intravenous injection of FDs at a dose of 120 mg/kg, the levels of FDs in the blood circulation and in the various organs were measured fluorometrically. FDs with a molecular weight lower than 20 kDa showed poor hepatic distribution (2.1—3.7% of dose/g tissue) due to their rapid elimination from the blood circulation. FDs with higher molecular weights were appreciably distributed in the liver (18.9—24.0% of dose/g tissue) and accumulated there over a long period, whereas the FD levels in the other organs were almost negligible a few days after injection. The hepatic mean residence time of FDs ranged from 22.5 to 28.1 d. Partial depolymerization of FDs which accumulated in the liver was observed within 10 d after administration. The hepatic uptake clearance of FDs was decreased with an increase in molecular weight. A marked molecular weight dependency was also seen in the urinary and fecal excretions of FDs. An appreciable dose-dependency was demonstrated in the hepatic uptake of FDs (MW = 40 kDa), as well. The amount of hepatic uptake as a function of dose showed saturation kinetics and was analyzed by a Michaelis-Menten type equation. The apparent values of K_m (dose) and V_{max} (hepatic level) estimated were 116 ± 5 mg/kg and 1.10 ± 0.05 mg/g tissue, respectively.

Key words fluorescein-labeled dextran; biodisposition; molecular weight; high performance size exclusion chromatography; mouse

Dextran is a polysaccharide consisting of glucose molecules coupled into long branched chains, mainly through a 1,6- and some through a 1,3- glucosidic linkages. Dextrans are colloidal, hydrophilic and water-soluble substances, inert in biological systems and do not affect cell viability. Because of these properties, dextrans have been used for many years as blood expanders to maintain or replace blood volume.[1)]

Macromolecular drug carrier systems have been developed in an attempt to alter the tissue localization of drugs such that the effects of the drugs at desired sites of action are enhanced. Many different biological macromolecules are available as carriers for drugs and enzymes.[2)] Among them, dextrans may serve as one of the most promising carrier candidates due to their excellent physicochemical properties and physiological acceptance.

Dextrans have been studied for use as a carrier system for a variety of therapeutic agents including antidiabetics, antibiotics, anticancer drugs, peptides and enzymes.[1,2)] It has been emphasized that the pharmacokinetics of polymeric drugs are governed to a large extent by the carrier moiety, but only a few reports which systematically evaluated the *in vivo* fate of dextrans have been seen so far.[3-5)]

In this paper, we report a systematic analysis of the biodisposition of fluoresceinisothiocyanate (FITC)-labeled dextrans with different molecular weights in the body of mice.

MATERIALS AND METHODS

Chemicals Fluorescein-labeled dextrans (FD-4 (MW = 4400), FD-10 (MW = 9400), FD-20 (MW = 18900), FD-40 (MW = 40500), FD-70 (MW = 69000), FD-150 (MW = 144000) and FD-500 (MW = 485000)) were purchased from Sigma Chemical Co., U.S.A. FDs were orange powders readily soluble in water. The degrees of substitution by FITC of dextrans varied with the preparation, ranging from 0.003 to 0.010 in FITC mol/glucose unit. All other chemicals and reagents were of the highest grade commercially available.

Animal Experiment Male ddy mice (20—30 g) were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). Mice were injected with FDs (120 mg/kg) in 0.2 ml of saline through the tail vein. At 0.5, 1, 2, 4, 6, 8 and 24 h after the administration, blood was collected from the *vena cava* under ether anesthesia, and various organs (liver, kidney, lung, spleen, brain, and gallbladder) were excised and weighed. Liver samples were further collected at 2, 3, 5, 10, 20 and 30 d after the drug administration. Each organ was homogenized on ice with a Potter-Elvehjem-type teflon homogenizer using a 19-fold volume of 0.1 M phosphate buffer (pH 7.4). For the gallbladder, a 119-fold volume of the buffer was used to make a homogenate.

Urine and feces samples were collected for 72 h after the administration of FDs. Feces were homogenized with a Waring Blender-type homogenizer using 19-fold volume of the buffer. The homogenate was centrifuged at 2500 rpm and the supernatant was filtered.

All samples of the organ homogenates, the feces filtrate and the urine were kept frozen at −20 °C until analyzed.

Analytical Method The amount of FDs in the biological samples was determined fluorometrically. The tissue homogenate or urine was diluted appropriately with the phosphate buffer, if necessary, and 40 μl of the sample solution was added to 3 ml of a 0.5 M Tris–HCl buffer (pH 8.0) containing 0.1% of sodium dodecylsulfate. After thorough mixing, the fluorescence at 520 nm was determined by the excitation at 495 nm. The blood space of each organ was determined by the tissue distribution data of FD-70 at 2 min after intravenous injection (120

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mg/kg). This value was used to correct for the presence of the drug in the residual blood remaining in the organ. The reproducibility of the assay procedure was acceptable, and recoveries are close to 100% on all media.

Estimation of Molecular Weight The molecular weight of FDs in biological samples was analyzed by high performance size exclusion chromatography (HPSEC). Each organ was first homogenized using a 3-fold volume of 0.1 M phosphate buffer (pH 7.4). Eighty microliters of 30% (w/v) trichloroacetic acid was added to 200 μl of the tissue homogenate (25% (w/v)). The mixture was vortexed and then centrifuged at 14000 rpm for 5 min. The supernatant (100 μl) was neutralized by the addition of 15 μl of 11% (w/v) of NaOH and was diluted with 85 μl of a mobile phase to make 200 μl. After filtration, 80 μl of the sample solution was injected into the HPLC system.

HPSEC was carried out using a Tosoh HPLC system (CCPD; Tokyo, Japan) equipped with a variable-wavelength fluorescence detector (RF-530, Shimadzu, Kyoto, Japan). The excitation and emission wavelengths were set at 495 and 520 nm, respectively. A 7.5 × 600 mm, TSKgel G3000SW column (Tosoh) was used at ambient temperature. The mobile phase was 0.2 M NaCl in 0.05 M phosphate buffer, pH 7.0 and the flow rate was 1.0 ml/min. Data analysis was done using a Tosoh super system controller SC-8010.

Data Analysis Assuming that the efflux of FDs from the liver is negligible during the time studied, the hepatic uptake clearance (CL_h) can be described as follows[5]:

$$CL_h = X_h(t_1)/AUC_{0\to t_1} \quad (1)$$

where $X_h(t_1)$ is the hepatic level of FDs at time t_1, and $AUC_{0\to t_1}$ is the area under the blood concentration–time curve up to that time.

The total clearance (CL_{tot}) and the renal clearance (CL_r) of FDs were calculated by the following equations:

$$CL_{tot} = D/AUC_{0\to\infty} \quad (2)$$

$$CL_r = (X_u)_\infty/AUC_{0\to\infty} \quad (3)$$

where D and $(X_u)_\infty$ are the dose of FDs and the total amount of FDs excreted into the urine, respectively. $AUC_{0\to\infty}$ is the AUC up to infinity.

Estimation of the pharmacokinetic parameters, elimination rate constant and volume of distribution, was done using a nonlinear least squares program (MULTI).[6] AUC was calculated by the trapezoidal method. The mean residence time (MRT) was calculated by the following equation.

$$MRT = AUMC_{0\to\infty}/AUC_{0\to\infty} \quad (4)$$

where $AUMC_{0\to\infty}$ is the area under the moment curve up to infinity.

RESULTS

Tissue Distribution of FDs with Various Molecular Weights Figure 1 shows the tissue distribution of FDs at 4 h after intravenous injection of the compound. The blood persistence of FDs increased with an increase in molecular weight. A molecular weight-dependency was also seen in the hepatic distribution of FDs. FD-4, FD-10 and FD-20 showed low hepatic levels (2.1—3.7% of dose/g tissue) due to their rapid elimination from the blood circulation. FDs with a molecular weight larger than 40 kDa attained high hepatic levels (18.9—24.0% of dose/g tissue) which were almost constant, regardless of their molecular weights.

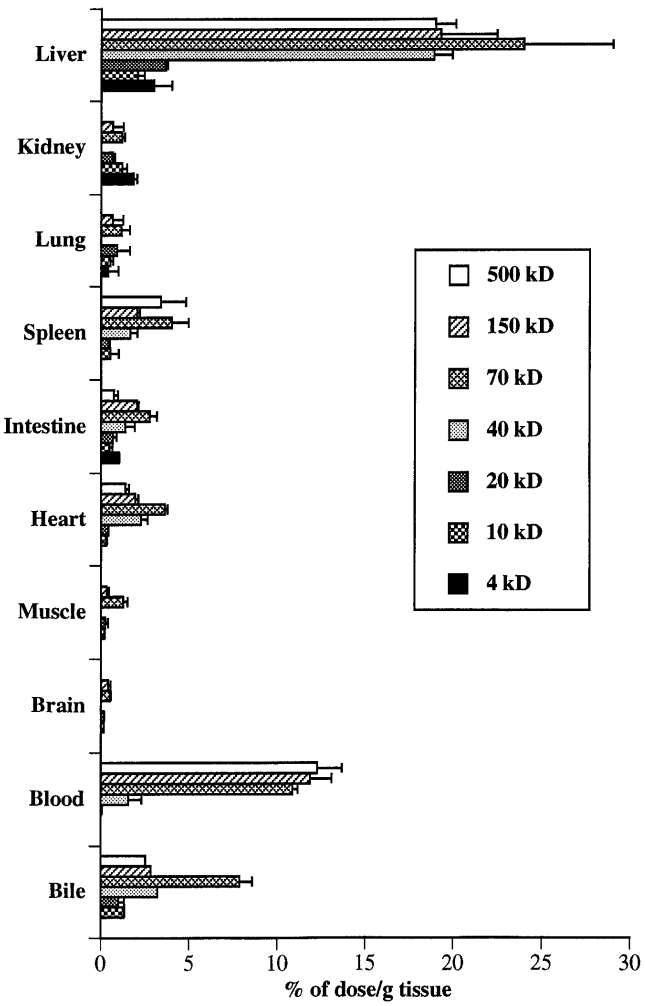

Fig. 1. Tissue Distribution of FDs Dependent on Molecular Weight at 4 h after Intravenous Injection (120 mg/kg) to Mice

Values are given as mean ± S.D. for groups of 3 mice.

Figure 2 shows the time profile of the tissue distribution of FD-40 after intravenous injection. A substantial level of FD-40 was found in the liver, and it reached a maximum at 4 h after injection. Allowing for the weight of the liver, the total amount of FD-40 recovered in this organ at 4 h after the injection was 33% of the dose. The amount of FD-40 excreted into the bile was diminished with the elapse of time, but was still detectable even after the compound was completely removed from the blood circulation (Fig. 2).

Elimination of FDs from Blood Circulation Among the FDs, FD-20, FD-40, FD-70 and FD-500 were chosen thereafter in order to examine their biodisposition, especially focusing on the hepatic distribution.

The blood concentration–time curves of FDs after intravenous injection are shown in Fig. 3. FDs exhibited

a monoexponential decline at the initial stage of the elimination. The calculated half-lives of FD-20, FD-40, FD-70 and FD-500 were 0.208, 0.831, 1.59 and 1.85 h, respectively. The volume of distribution was also calculated, with results listed in Table 1.

Excretion of FDs into Urine and Feces The time profiles of cumulative urinary excretions of FDs are depicted in Fig. 4. The urinary excretion was terminated within 12 h, and 64.3% (FD-20), 34.9% (FD-40), 10.2% (FD-70) or 2.4% (FD-500) of dose was excreted totally in urine. Figure 5 shows the time profiles of cumulative fecal excretions of FDs. The amounts of FDs excreted in feces were 9—16% of the dose at 72 h after the injection, but continued to increase gradually in the case of FD-70 and FD-500.

Hepatic Distribution and Hepatic Uptake Clearance of FDs Figure 6 shows the time profiles of hepatic levels of FDs after intravenous injection. The levels reached a maximum at 2—8 h and remained almost constant within 24 h.

Assuming the efflux of FDs from the liver was negligible during the time studied, as shown in Fig. 6, the hepatic

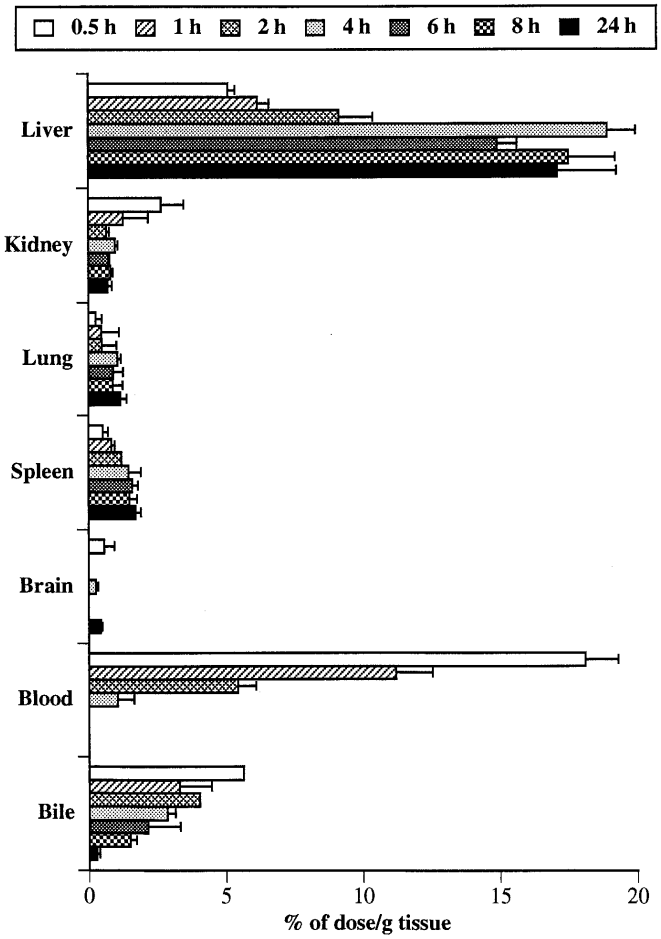

Fig. 2. Tissue Distribution of FD-40 after Intravenous Injection (120 mg/kg) to Mice

Values are given as mean ± S.D. for groups of 3 mice.

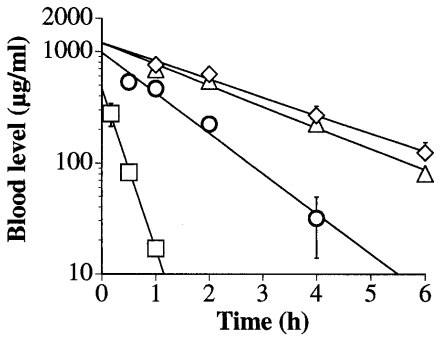

Fig. 3. Blood Level of FDs after Intravenous Injection (120 mg/kg) to Mice

□, FD-20; ○, FD-40; △, FD-70; ◇, FD-500. Values are given as mean ± S.D. for groups of 3 mice.

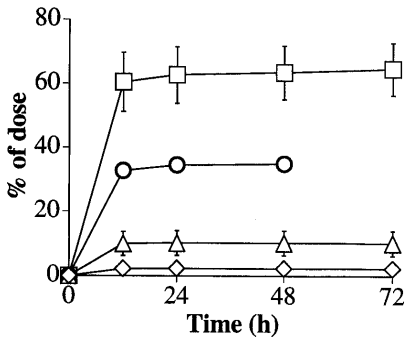

Fig. 4. Cumulative Urinary Excretion of FDs after Intravenous Injection (120 mg/kg) to Mice

□, FD-20; ○, FD-40; △, FD-70; ◇, FD-500. Values are given as mean ± S.D. for groups of 4 mice.

Table 1. Pharmacokinetic Parameters of FDs after Intravenous Injection (120 mg/kg) to Mice

Parameters	FDs			
	FD-20	FD-40	FD-70	FD-500
Elimination rate constant (h^{-1})	3.34 ± 0.13	0.834 ± 0.079	0.437 ± 0.033	0.375 ± 0.024
Biological half-life (h)	0.208 ± 0.008	0.831 ± 0.079	1.59 ± 0.12	1.85 ± 0.12
Volume of distribution (ml/kg body weight)	255 ± 1	122 ± 0.1	101 ± 0.1	99.8 ± 0.1
Total clearance (ml/h/kg body weight)	852 ± 33	102 ± 10	44.1 ± 3.3	37.4 ± 2.4
Hepatic clearance				
(ml/h/g tissue)	0.716 ± 0.035	0.442 ± 0.016	0.258 ± 0.019	0.231 ± 0.010
(ml/h/kg body weight)[a]	56.1 ± 13.2	33.0 ± 7.7	20.2 ± 4.9	18.1 ± 4.2
Total urinary excretion (% of dose)	64.4 ± 8.2	34.9 ± 0.8	10.2 ± 3.8	2.4 ± 0.2
Renal clearance (ml/h/kg body weight)	549 ± 73	35.6 ± 3.6	4.50 ± 1.71	0.898 ± 0.094
Mean residence time in blood (h)	0.348	1.16	1.89	2.13
Mean residence time in liver (d)	23.5	22.5	25.6	28.1

a) The ratio of the liver weight to the body weight was 0.0783 ± 0.018 in mice with an average body weight of 24.3 ± 2.9 g (n = 41). *b*) Values are given as mean ± S.D.

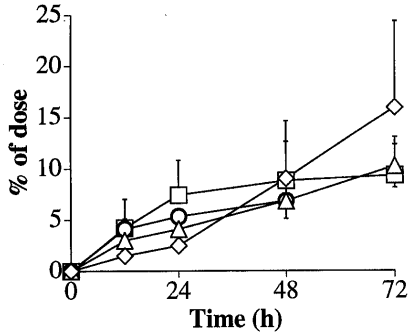

Fig. 5. Cumulative Fecal Excretion of FDs after Intravenous Injection (120 mg/kg) to Mice

□, FD-20; ○, FD-40; △, FD-70; ◇, FD-500. Values are given as mean±S.D. for groups of 4 mice.

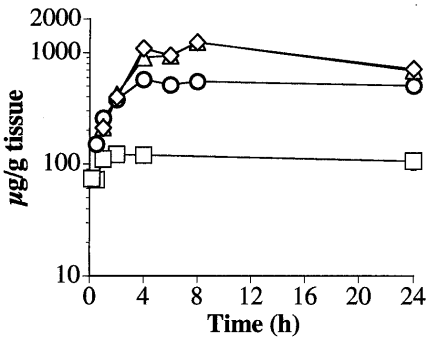

Fig. 6. Hepatic Level of FDs after Intravenous Injection (120 mg/kg) to Mice

□, FD-20; ○, FD-40; △, FD-70; ◇, FD-500. Values are given as mean for groups of 3 mice. Bars for values of S.D. were smaller than the symbols.

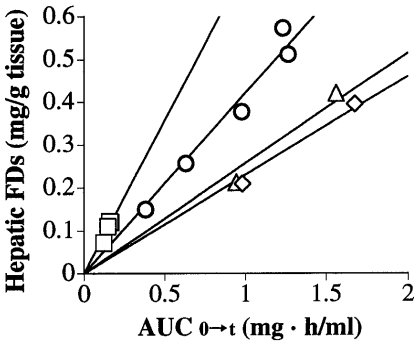

Fig. 7. Relationship between Hepatic FDs at Time t and $AUC_{0\to t}$ after Intravenous Injection (120 mg/kg) to Mice

□, FD-20; ○, FD-40; △, FD-70; ◇, FD-500.

uptake clearance was calculated by Eq. 1 as described in the experimental section. The integration plot, that is, the relationship between the hepatic level of FDs at time t and the $AUC_{0\to t}$ after intravenous injection in mice, was plotted as shown in Fig. 7. The slope of the linear regression line gave the hepatic uptake clearance, which was summarized in Table 1.

Hepatic Accumulation of FDs FDs were appreciably distributed in the liver and accumulated there over a long period (Fig. 8), whereas the levels of FDs in the other organs were almost negligible within a few days after injection. At 30 d after the injection of FDs, 8—13% of the dose was still detectable in the liver. The hepatic mean residence time was 22.5—28.1 d (Table 1).

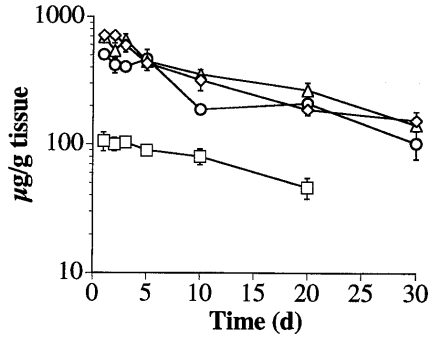

Fig. 8. Hepatic Level of FDs after Intravenous Injection (120 mg/kg) to Mice

□, FD-20; ○, FD-40; △, FD-70; ◇, FD-500. Values are given as mean±S.D. for groups of 3 mice.

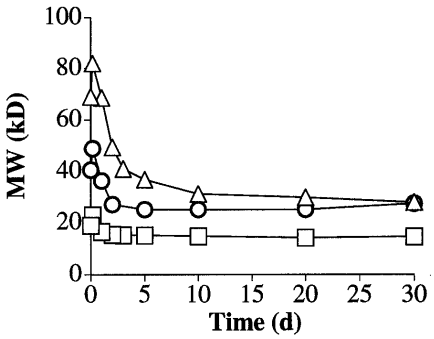

Fig. 9. Hepatic Degradation of FDs after Intravenous Injection (120 mg/kg) to Mice

□, FD-20; ○, FD-40; △, FD-70. Values are given as mean for groups of 3 mice. Bars for values of S.D. were smaller than the symbols.

Molecular weight distributions of FDs having accumulated in the liver were examined by HPSEC. Initial degradations of FD-20 to 14 kDa, of FD-40 to 25 kDa, and of FD-70 to 31 kDa were observed within 10 d after administration (Fig. 9).

Dose-Dependent Hepatic Distribution of FD-40 Figure 10 shows the tissue distribution of FD-40 at 4 h after intravenous injection at various doses. The blood persistence of FD-40 increased with an increase in dose due to the saturation in the hepatic uptake. The amounts of FD-40 recovered in the liver at 4 h after administration were 33, 19, 7.5 and 3.2% of dose/g tissue at doses of 24, 120, 600 and 1200 mg/kg, respectively.

Figure 11 shows the cumulative urinary and fecal excretions of FD-40 at 48 h after intravenous injection at various doses. Urinary excretion was terminated within 12 h, and 31.1% (24 mg/kg), 34.9% (120 mg/kg), 47.3% (600 mg/kg) and 41.7% (1200 mg/kg) of the dose was excreted in urine. As can be seen in Fig. 11, the urinary excretion ratio to dose was slightly increased with an increasing dose, but did not exceed 50% of the dose. The amounts of FDs excreted in feces were not markedly changed, being 8.4, 6.8, 10.0 and 10.0% of the dose at doses of 24, 120, 600 and 1200 mg/kg, respectively.

The relationship between the hepatic level (X_h) against the dose was analyzed by the Michaelis-Menten type equation. Figure 12 shows the Lineweaver-Burk plot for the dose-dependent hepatic distribution of FD-40. The maximum hepatic level (V_{max}) and the Michaelis constant

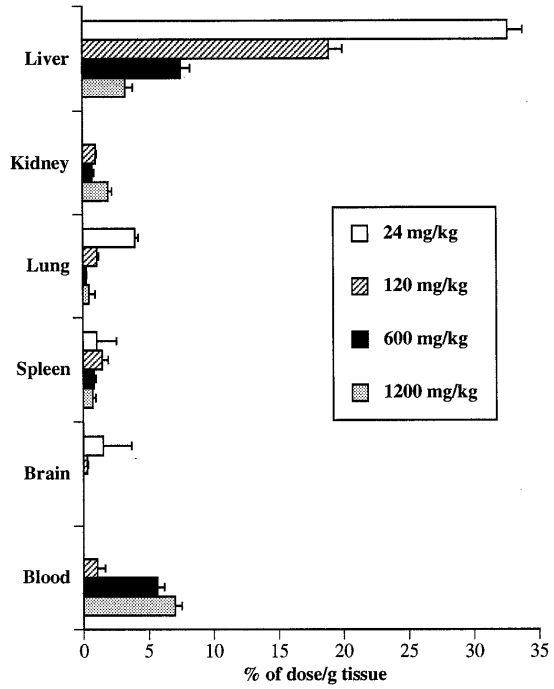

Fig. 10. Dose Dependent Tissue Distribution of FD-40 at 4h after Intravenous Injection to Mice

Values are given as mean±S.D. for groups of 3 mice.

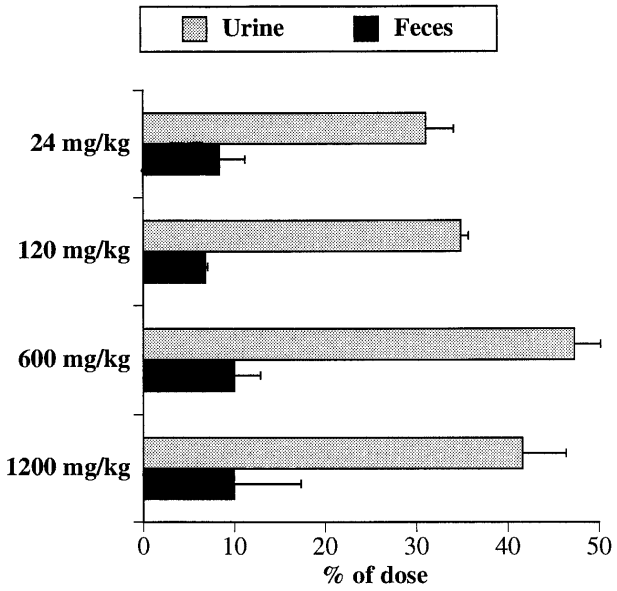

Fig. 11. Effect of Dose on the Excretion of FD-40 after Intravenous Injection to Mice

Values are given as mean±S.D. for groups of 3 mice.

(K_m) were estimated to be 1.10±0.05 mg/g tissue and 116±5 mg/kg, respectively.

DISCUSSION

Pharmacokinetics of FDs Dependent on Molecular Weight As can be seen in Figs. 1 and 3, the residence time of FDs in the blood circulation was markedly prolonged with increasing molecular weight. In contrast, the values of CL_{tot} were decreased with an increase in molecular weight (Table 1). This was due not only to the

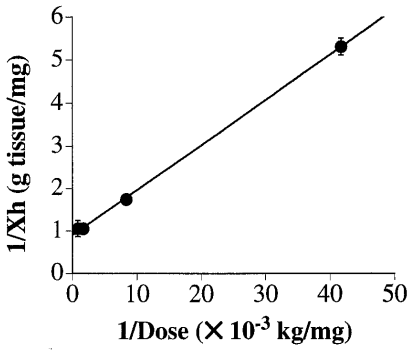

Fig. 12. Lineweaver-Burk Plot for Dose-Dependent Hepatic Distribution of FD-40 at 4 h after Intravenous Injection to Mice

Values are given as mean±S.D. for groups of 3 mice.

diminished values of the elimination rate constant, but also to the decrease in the values of volume of distribution (V_d). The V_d values of FDs with a higher molecular weight approached a total blood volume (approximately 100 ml/kg body weight). On the other hand, FD-20 showed a high V_d value (255 ml/kg body weight), suggesting advanced distribution into the interstitial space.

Urinary excretion terminated within 12 h and was drastically diminished with increasing molecular weight (Fig. 4). Dextran, with a molecular weight lower than 15000, is mainly eliminated by the kidney. Larger molecules with a molecular weight above 50000 are practically not excreted *via* this route.[7,8] A similar observation was made in our studies with FDs (Fig. 4, Table 1).

Hepatic Uptake of FDs Dependent on Molecular Weight A distinct difference in hepatic distribution was seen between FDs with a molecular weight of less than 20000 and those with a molecular weight larger than 40000 (Figs. 1 and 6). The hepatic accumulation of FDs reached a maximum at the molecular weight of 70000 and was maintained constant above this value. Although the prolonged retention of FDs with larger molecular weights in the blood circulation apparently caused higher hepatic accumulation, it would be interesting to see whether the hepatic uptake process is dependent on molecular weight.

Therefore, the hepatic uptake clearance of FDs was calculated by an integration plot (Fig. 7). As shown in Table 1, the values of CL_h for FD-20, FD-40, FD-70 and FD-500 were 56.1, 33.0, 20.2 and 18.1 ml/h/kg body weight, respectively. It was found that the hepatic uptake clearance of FDs was decreased with an increase in molecular weight. Stock *et al.* reported the same phenomena after measuring the transport in the microcirculation of isolated perfused rat liver; the permeability of FDs into the hepatocytes was dependent on the molecular weight.[9]

Hepatic Accumulation of FDs Figure 2 shows that FDs are rapidly distributed into such tissues as liver, kidney, lung and spleen after intravenous injection. However, FDs were no longer detectable in these tissues, except the liver, 1—2 d after the administration. An extended stay of FDs was demonstrated in the liver over a long period of time (30 d); the values of mean residence time of FD-20, FD-40, FD- 70 and FD-500 were estimated to be 23.5, 22.5, 25.6

and 28.1 d, respectively, which were independent of the molecular weight (Fig. 8 and Table 1).

Earlier reports indicated that dextran with a molecular weight above 50000 not excreted *via* the kidneys, was, however, completely eliminated from the blood stream, and that dextran uptake by the liver was observed, followed by continuous and complete elimination from the liver tissue.[8] Chemical methods, such as anthrone reaction, that have been used for the assay of dextran in tissues are not as reliable as those used for measuring dextran in blood and urine. In this study, the hepatic distribution of FDs was determined fluorometrically, and was confirmed using a specific HPSEC method.

It has been well recognized that large numbers of dextran-splitting anaerobic bacteria, belonging to the Bacteroides genus, occur in the colon and account for the dextran-degrading capacity of feces.[10] In this study, we also found that FD-40 was recovered in 0—12 h feces as completely depolymerized fractions after oral administration to mice at a dose of 120 mg/kg. The HPSEC chromatogram of the degradation products was almost identical to that obtained after the *in vitro* action of dextranase on FD-40 (data not shown).

A dextran-splitting enzyme was found in tissues such as the spleen, intestine, liver and kidney.[11-13] In our *in vitro* experiment, FD-40 was degraded in homogenates of either the intestine or feces after 24-h incubation at pH 6.0 and 37 °C. Since the liver homogenate had very low dextran-splitting activity, it took a long incubation time of several days to demonstrate the enzymatic degradation of FD-40 in the *in vitro* experimental system (Kaneo Y, unpublished data). The depolymerization of FDs in the liver are clearly demonstrated also in *in vivo* animal experiments, as shown in Fig. 9.

Dose-Dependent Hepatic Uptake of FDs A marked dose-dependency was found in the hepatic uptake of FD-40 in mice (Fig. 10). More than 70% of the FD-40 injected intravenously was recovered in the liver at the lowest dose of 24 mg/kg. As shown in Fig. 11, the total urinary excretion of FD-40 was almost unchanged when the dose was increased from 24 to 1200 mg/kg. This indicated that the renal clearance was not appreciably dependent on the dose. On the other hand, the maximum hepatic level of FD-40 was attained at 4 h and the efflux of the compound from the liver was negligible during the several hours following. Therefore, in the case of FD-40, the amount of hepatic uptake as a function of dose showed saturation kinetics and was simply analyzed by a Michaelis-Menten type equation (Fig. 12). The apparent values of K_m (dose) and V_{max} (hepatic level) estimated were 116 ± 5 mg/kg and 1.10 ± 0.05 mg/g tissue, respectively.

Both parenchymal and Kupffer cells were shown to participate in the removal of dextran by histological studies in mice.[14] We examined the hepatic cellular localization of FD-70 in rats by a method involving collagenase perfusion followed by Percoll centrifugation. FD-70 was predominantly taken up by the nonparenchymal cells, with the cell uptake (μg/mg protein) being 4 times higher than that of the parenchymal cells.[15]

Endocytosis is a widespread process of cell surface invagination and subsequent internalization of plasma membrane as vacuoles, which is associated with transport of extracellular solutes into the cell. FITC-labeled dextrans have been used as one of the inert fluid-phase markers to characterize the transcellular vesicular transport by fluid-phase endocytosis.[16,17] Polyvinylpyrrolidone, a synthetic, metabolically stable hydrophilic polymer, is taken up solely by fluid-phase endocytosis. The hepatic uptake clearance of polyvinylpyrrolidone in rats was reported to be 0.008 ml/h/g tissue,[18] which was much smaller than that of FDs (0.231—0.716 ml/h/g tissue) obtained in this study (Table 1).

Watanabe *et al.*[19,20] observed the dose-dependent hepatic uptake of fractionated [^{3}H]heparin in rats. They also pointed out that the uptake clearance was much larger than that reported for polyvinylpyrrolidone. A recent work reported that the hepatic uptake of dextran was inhibited by galactosyl-bovine serum albumin, suggesting that dextran might interact with the asialoglycoprotein receptor.[21] Groman *et al.*,[22] however, failed to demonstrate the interaction of dextran with the receptor, which was isolated from rat liver. On the other hand, Mehvar *et al.*[23,24] reported that a marked reduction in the hepatic accumulation of dextran was induced in diabetic rats and revealed that it was due to the hyperglycemia. It was also reported that a hypertonic medium reduces the endocytosis of macromolecules.[25]

In this study, the hepatic accumulation and disposition of FDs with different molecular weights were demonstrated in mice. Taking into account that the hepatic uptake of dextran is slow and not easily saturable with a large V_{max} value, we cannot rule out the possibility of the existence of some nonspecific binding site(s) which is common to polysaccharides. Further studies on the mechanism of hepatic uptake of polysaccharides, including dextrans, are needed in the future.

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