ELSEVIER

International Journal of Pharmaceutics 201 (2000) 59–69

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Pharmacokinetics and biodisposition of fluorescein-labeled arabinogalactan in rats

Yoshiharu Kaneo [a,*], Takashi Ueno [a], Tetsuro Tanaka [a], Hiroki Iwase [a], Yasunori Yamaguchi [b], Tomochika Uemura [a]

[a] *Laboratory of Biopharmaceutics, Faculty of Pharmacy and Pharmaceutical Sciences, Fukuyama University Fukuyama, Hiroshima, 729-0292, Japan*

[b] *Laboratory of Animal Cell Technology, Department of Biotechnology, Faculty of Technology, Fukuyama University, Fukuyama, Hiroshima, 729-0292, Japan*

Received 17 November 1999; received in revised form 21 February 2000; accepted 6 March 2000

Abstract

Fluorescein-labeled arabinogalactan (FA) was prepared by the reaction with FITC in methyl sulphoxide according to the method of deBelder and Granath. A systemic kinetic analysis of FA in rats was carried out by using a specific high-performance size-exclusion chromatography. Intravenously administered FA was rapidly eliminated from the blood circulation followed by an appreciable distribution to the liver and kidney. FA was accumulated in these organs over a long period whereas negligible levels of FA were detected in the other organs. A marked dose-dependency was seen in the hepatic uptake of FA which was markedly reduced by coinjected asialofetuin whereas the renal uptake of FA was not altered. Measurement of the hepatocellular localization demonstrated the overwhelming distribution of FA in the parenchymal liver cell fraction. Furthermore, the microscopic examination revealed FA that was effectively endocytosed by the parenchymal liver cells. These results suggested that FA which is bound to the asialoglycoprotein receptor with a high affinity is subsequently internalized to the hepatocyte via receptor-mediated endocytosis. FA was partially activated by periodate oxidation in order to acquire aldehyde groups to which guest molecules can be bound. A 12.5% oxidized arabinogalactan keeping a hepatocellular targetability showed a good conjugating reactivity to guest molecules via Schiff-base formation or by reductive amination. It was suggested that arabinogalactan can serve as a potential carrier for the delivery of enzymes and drugs to the parenchymal liver cells via the asialoglycoprotein receptor.

Keywords: Arabinogalactan; FITC; Biodisposition; Pharmacokinetics; High performance size exclusion chromatography; Fluorescence microscopy; Glycotargeting; Asialoglycoprotein receptor; Hepatotropic carrier; Rat

1. Introduction

Glycotargeting relies on carrier molecules possessing carbohydrates that are recognized and internalized by cell surface mammalian lectins

* Corresponding author. Tel.: +81-849-362111; fax: +81-849-362024.

E-mail address: kaneo@fupharm.fukuyama-u.ac.jp (Y. Kaneo)

0378-5173/00/$ - see front matter
PII: S0378-5173(00)00405-1

(Meijer et al., 1992; Monsigny et al., 1994; Wadhwa and Rice, 1995). Macromolecular carriers including asialoglycoproteins of natural origin, synthesized neoglycoproteins and synthetic polymers modified to contain galactose residues were intensively examined for the purpose of hepatic targeting (DiStefano and Fiume, 1997).

Arabinogalactan is a complex, highly branched, water-soluble polysaccharide found in larchwood. The basic building units for the majority of arabinogalactans are arabinose and galactose in a ratio of approximately 1:6 (Adams and Douglas, 1963; Odonmazig et al., 1994). Uhlenbruck et al. (1987) found that the experimental liver metastases in mice were inhibited by blocking hepatocyte lectins with arabinogalactan infusion. Recently, a superparamagnetic iron oxide colloid covered with arabinogalactan has been developed as a hepatic selective magnetic resonance contrast agent (Josephson et al., 1990; Weissleder et al., 1990).

The highly branched structure and the presence of numerous terminal galactose residues of arabinogalactan are responsible for its binding to the asialoglycoprotein receptor (Groman et al., 1994). Since arabinogalactan is not immunogenic and is a very safe compound (Naim and vanOss, 1992; Groman et al., 1994), it is attractive for the receptor-mediated delivery of enzymes and drugs to the liver parenchymal cells (Enriquez et al., 1995; Cui et al., 1997).

It has been emphasized that the pharmacokinetics of the polymeric drugs are governed to a large extent by the carrier moiety, but only a few reports which evaluated the in vivo fate of arabinogalactan have been seen so far. We report a systemic kinetic analysis of fluorescein-labeled arabinogalactan in rats by using a specific high-performance size-exclusion chromatography (HPSEC).

2. Materials and methods

2.1. Chemicals

Arabinogalactan (from larch wood; A 2012) and asialofetuin (type 1, from fetal calf serum; A 4781) were purchased from Sigma, St Louis, MO. Fluorescein isothiocyanate isomer I (FITC) was obtained from Wako Pure Chemical, Osaka, Japan. All other chemicals and reagents were of the highest grade commercially available.

2.2. Preparation of FITC-labeled arabinogalactan (FA)

FITC-labeled arabinogalactan (FA) was prepared by the method of deBelder and Granath (1973). Arabinogalactan (1 g) was dissolved in methyl sulphoxide (10 ml) containing 3 drops of pyridine. FITC (400 mg) was added, followed by dibutyltin dilaurate (20 mg), and the mixture was heated for 2 h at 95°C. After several precipitations in ethanol to remove free dye, the fraction of FA was dried in vacuo at 80°C. The FA was further purified by size-exclusion chromatography on Sephadex G-25, and then freeze-dried. The FA sample was dissolved in 25 mM borate buffer (pH 9.0), and the absorbance at 495 nm was measured. The fluorescein content of FA was estimated using the calibration curve of fluorescein sodium (uranine).

2.3. Preparation of polyaldehyde arabinogalactan

Arabinogalactan (2 g) was oxidized with $NaIO_4$ solution (0.123 M, 100 ml) at 4°C in the dark for 24 h with stirring. This represented a ratio of 1 mole periodate added per mole glycosidic residues in the arabinogalactan and was considered a 100% oxidation. Different preparations were also produced in which the degree of oxidation was 12.5, 25 or 50%. The oxidized arabinogalactan was then dialyzed extensively against water at 4°C in the dark for 48 h and lyophilized. The aldehyde content of the oxidized arabinogalactan was determined by the alkaline iodine titration method (the Romijin method).

A batch (500 mg) of polyaldehyde arabinogalactans of each degree of oxidation in 10 ml of 0.5 M phosphate buffer (pH 7.4) was reduced by the slow addition of $NaBH_4$ (1.7 g/l mM NaOH 5 ml). After standing for 18 h in the dark, the solution was acidified by adding of concentrated HCl for 5 min and was neutralized by adding of

NaOH. The reduced polyaldehyde arabinogalactan was then dialyzed extensively against water at 4°C in the dark for 48 h and lyophilized. FITC was introduced to the reduced polyaldehyde arabinogalactan by the same method described above.

2.4. Animal experiment

2.4.1. Plasma level

Male Wistar rats weighing 200–300 g were fasted for 16 h prior to the experiments. Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and were kept on a 40°C plate. Rats were injected with a single dose of FA in 0.2 ml of saline through the jugular vein. Blood samples (0.3 ml) were obtained periodically through a cannulated femoral artery after the drug administration. The samples were centrifuged in a microcentrifuge for 5 min, and the resultant serum was harvested. At the end of the experiment, liver was excised and treated as described later.

2.4.2. Tissue distribution

Rats were injected with FA (12 mg/kg) in 0.2 ml of saline through the tail vein. At 2 h after the administration, blood was collected from the vena cava under ether anesthesia and various organs (liver, kidney, lung, spleen, and brain) were excised and weighed. Liver and kidney samples were collected periodically until 14 day after the drug administration. Each organ was homogenized on ice with a Potter–Elvehjem-type teflon homogenizer using a 3-fold volume of 0. 1 M phosphate buffer (pH 7.4).

2.4.3. Separation of liver cells

Rats were injected with FA (12 mg/kg) or FITC-labeled dextran (FD-70; MW, 70 kDa) through the tail vein. At 2 h after the drug administration, the liver was perfused with collagenase to isolate the liver cells according to the method of Seglen (1976), and parenchymal and nonparenchymal cells were separated by Percoll centrifugation method. Each fraction was homogenized using an appropriate volume of 0.1 M phosphate buffer (pH 7.4).

2.4.4. Bile excretion

Rats were anesthetized by sodium pentobarbital. A polyethylene catheter (PE-10) was inserted into the bile duct. Bile samples were collected successively until 8 h after administration of FA (12 mg/kg) through the jugular vein.

2.4.5. Urinary and fecal excretions

Urine and feces samples were collected for 48 h after the administration of FA (12 mg/kg) through the tail vein. Urine samples were filtered and diluted appropriately. Feces were homogenized with a Waring Blender-type homogenizer using 9-fold volume of the buffer. The homogenate was centrifuged at 2500 rpm and the supernatant was filtered.

2.4.6. Fluorescence microscopic examination

Rats were injected with FA (120 mg/kg) in 0.2 ml of saline through the tail vein. The liver was excised periodically under ether anesthesia. The liver tissues were fixed overnight in 10% formaldehyde in neutral phosphate buffer in the dark at room temperature. After dehydration in EtOH the tissues were embedded in paraffin for the preparation of thin sections. Specimens were examined with a Hitachi transmitted light fluorescence microscope.

2.5. Analytical method

2.5.1. High-performance size-exclusion chromatography (HPSEC)

FA in biological samples was analyzed by HPSEC. A hundred microliters of H_2O and 80 μl of 30% (w/v) trichloroacetic acid were added to 100 μl of plasma. The mixture was vortexed and then centrifuged at 14 000 rpm for 5 min. The supernatant (100 μl) was neutralized by 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. In biological samples other than plasma, 200 μl of the sample solution was used without addition of 100 μl of H_2O.

HPSEC was carried out using a Tosoh HPLC system (CCPD; Tokyo, Japan) equipped with a

variable-wavelength fluorescent detector (RF-530, Shimadzu, Kyoto, Japan). The excitation and emission wavelengths were set at 495 and 520 nm, respectively. A 7.8 × 300 mm, TSKgel G3000SWXL 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.

2.5.2. Fluorometric determination

The amount of FA in the feces samples was determined fluorometrically. The filtrate (40 μl) of the homogenate supernatant was added to 3 mI of a 0.5 M Tris-HCl buffer (pH 8.0) containing 0.1% of sodium dodecylsulfate. After thorough mixing, the fluorescence at 520 mn was determined by the excitation at 495 nm.

2.6. Data analysis

Assuming that the efflux of FA from the liver is negligible during the time studied, the hepatic uptake clearance (CL_h) can be described as follows (Takakura et al., 1990):

$$CL_h = X_h(t_1)/AUC_{0\rightarrow t1} \quad (1)$$

where $X_h(t_1)$ is the hepatic level of FA at time t_1, and $AUC_{0\rightarrow t1}$ 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 FA were calculated by the following equations:

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

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

where D and $(X_u)_\infty$ are the dose of FA and the total amount of FA excreted into the urine, respectively. $AUC_{0\rightarrow\infty}$. 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) (Yamaoka et al., 1981). AUC was calculated by the trapezoidal method.

The mean residence time (MRT) was calculated by the following equation.

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

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

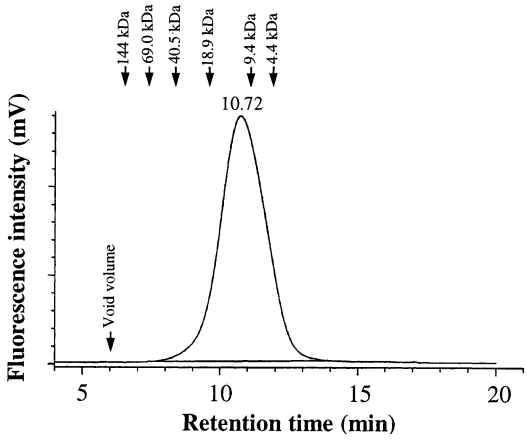

Fig. 1. Analysis of FITC-labeled arabinogalactan using high performance size exclusion chromatography (HPSEC). HPSEC was carried out using a HPLC system equipped with a variable-wavelength fluorescence detector. The excitation and emission wavelengths were set at 495 and 520 nm, respectively. A 7.8 × 300 mm, TSKgel G3000SWXL column 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. The arrows indicate the retention time of the FITC-labeled dextran standards. The void volume was determined using blue dextran (2000 kDa).

3. Results

3.1. Chromatographic analysis of FA

Size-exclusion chromatography of FA which was monitored by fluorescence detection (λ_{ex}, 495 nm, λ_{em}, 520 nm) yielded a single peak as shown in Fig. 1. A strong linear relationship in the wide range was observed between the peak area and the amount of FA injected. The sensitivity of the assay was close to 25 ng as FA injected.

The peak of FA corresponded to a molecular weight of 12.6 kDa using FITC-labeled dextran standards (4.4–144 kDa). A molecular weight of intact arabinogalactan determined by HPSEC with a differential refractometer was 12.8 kDa, suggesting that the FITC-substitution has no influence on the molecular weight estimation. The

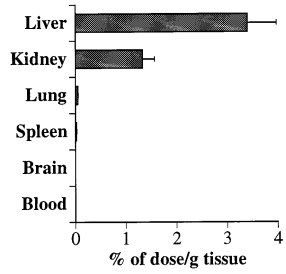

Fig. 2. Tissue distribution of FA at 2 h after intravenous injection (12 mg/kg) to rats. Values are given as mean ± S.D. for groups of three rats.

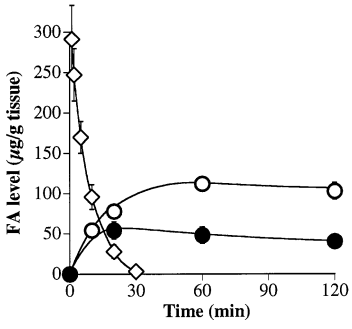

Fig. 3. Tissue levels of FA after intravenous injection (12 mg/kg) to rats. ◇, plasma; ○, liver; ●, kidney. Values are given as mean ± S.D. for groups of three rats.

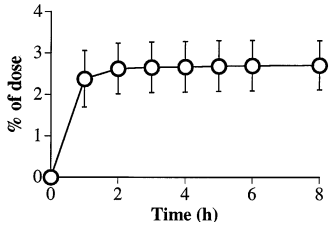

Fig. 4. Cumulative biliary excretion of FA after intravenous injection (12 mg/kg) to rats. Values are given as mean ± S.D. for groups of three rats.

degree of substitution by FITC was estimated to be 0.0064 FITC mol/sugar unit; one FA molecule contained on the average 1.6 FITC molecules.

Molecular weight determination of arabinogalactan by low-angle laser light scattering in the static mode in conjunction with high performance size exclusion chromatography (HPSEC-LALLS) generated a molecular weight of 39.7 ± 0.3 kDa (Fig. 1).

3.2. Tissue distribution of FA

Fig. 2 shows the tissue distribution of FA at 2 h after intravenous injection of FA to rats at a dose of 12 mg/kg. Relatively high levels of FA in liver and kidney were observed. FA was slightly distributed into lung and spleen but was not detected in brain and blood.

Fig. 3 shows the time profile of the distribution of FA in plasma, liver and kidney after intravenous injection. FA was rapidly eliminated from the blood circulation. Appreciable levels of FA were found in the liver and kidney, and they reached a maximum at 30–60 min after injection. Allowing for the weights of the liver and kidney, the total amounts of FA recovered in these organs at 60 min after the injection was 25 and 2.6% of the dose, respectively.

3.3. Excretion of FA into bde, urine and feces

The time profile of cumulative biliary excretion of FA is depicted in Fig. 4. A large portion of the biliary FA was recovered within the first 1 h and 2.7% of the dose was excreted totally in the bile. FA was kept intact without any degradation in the bile.

The time profiles of cumulative urinary and fecal excretions of FA are illustrated in Fig. 5.The urinary excretion was almost terminated within 12 h and 50% of the dose was excreted totally in urine. The amount of FA excreted in feces was 12% of the dose at 48 h after injection, but continued to increase gradually. FA was excreted as an intact form in urine but as a degraded product with low molecular weights in feces.

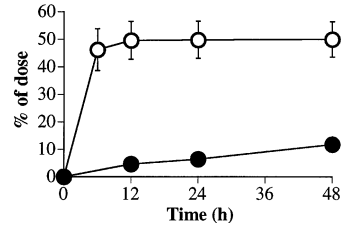

Fig. 5. Cumulative urinary (○) and fecal (●) excretions of FA after intravenous injection (12 mg/kg) to rats. Values are given as mean ± S.D. for groups of three rats.

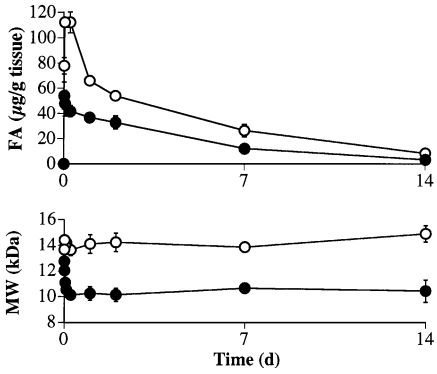

Fig. 6. Hepatic (○) and renal (●) level-time profiles and the corresponding molecular weights (MW) of FA after intravenous injection (12 mg/kg) to rats. Values are given as mean ± S.D. for groups of three rats.

Table 1
Pharmacokinetic parameters of FA after intravenous injection (12 mg/kg) to rats

Elimination rate constant (h^{-1})	7.59 ± 0.28
Biological half-life (min)	5.46 ± 0.20
Volume of distribution (ml/kg body weight)	36.9 ± 0.6
Total clearance (ml/h per kg body weight)	270 ± 45
Hepatic uptake clearance (ml/h per g tissue)	1.67 ± 0.05
(m/h per kg body weight)[a]	47.1 ± 8.8
Renal uptake clearance (ml/h per g tissue)	0.968 ± 0.108
(ml/h per kg body weight)[a]	6.67 ± 1.52
Urinary clearance (ml/h per kg body weight)[b]	135 ± 28
Biliary clearance (ml/h per kg body weight)[c]	7.33 ± 2.01
Mean residence time in plasma (min)	7.10
Mean residence time in liver (day)	5.67
Mean residence time in kidney (day)	4.74

[a] The ratios of the liver and kidney weights to the body weight were 0.0282 ± 0.0052 and 0.00689 ± 0.00137, respectively, in rats with an average body weight of 235 ± 31 g ($n = 20$).

[b] Total urinary excretion of FA was 50.0 ± 6.4% of the dose.

[c] Total biliary excretion of FA was 2.71 ± 0.59% of the dose.

Values are given as mean ± S.D.

3.4. Hepatic and renal accumulation of FA

FA was appreciably distributed in the liver and kidney, and accumulated there over a long period (Fig. 6), whereas negligible levels of FA were observed in the other organs (Fig. 2). At 14 days after the injection of FA, 3.3% of the dose was still detectable in the liver where FA existed only in the intact form (12.6 kDa). On the other hand, an initial degradation of FA to 10 kDa was observed in the kidney (Fig. 6).

3.5. Pharmacokinetic parameters of FA

Pharmacokinetic parameters of FA are summarized in Table 1. FA was rapidly disappeared from the blood circulation; the biological half life was 5.46 min. Total clearance of FA was 270 ml/h per kg body weight, to which urinary clearance contributed 50%, hepatic uptake clearance 17.4%, renal uptake clearance 2.5% and biliary clearance 2.7%, respectively. The sum of the values for these clearances was 196 ml/h per kg body weight, comprising 73% of the total clearance. Mean residence times in liver and kidney were 5.67 and 4.74 days, respectively (Table 1).

3.6. Dose-dependent pharmacokinetics of FA

Fig. 7 shows the plasma levels of FA after intravenous injection at various doses. The blood persistence of FA was increased with an increase in dose. The plasma concentration–time curves

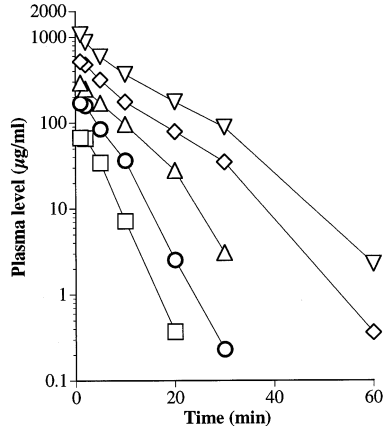

Fig. 7. Plasma level of FA after intravenous injection to rats at various doses. □, 3 mg/kg; ○, 6 mg/kg; △, 12 mg/kg; ◇, 24 mg/kg; ▽, 48 mg/kg.

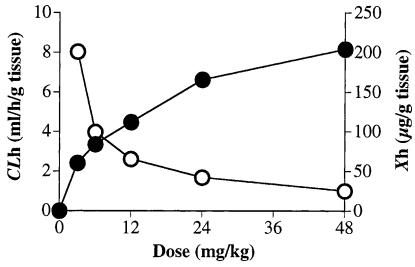

Fig. 8. Hepatic uptake clearance (CL_h, ○) and hepatic level (X_h, ●) of FA after intravenous injection to rats at various doses.

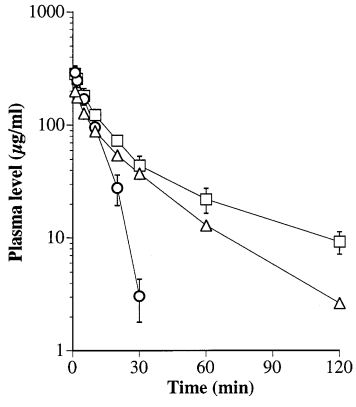

Fig. 9. Effect of coadministered asialofetuin or arabinogalactan on the plasma level of FA after intravenous injection (12 mg/kg) to rats. ○, FA; □, FA and asialofetuin (240 mg/kg); △ FA and arabinogalactan (240 mg/kg). Values are given as mean ± S.D. for groups of three rats.

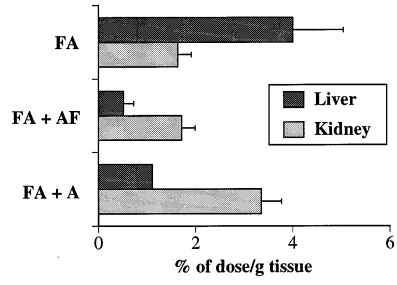

Fig. 10. Effect of asialofetuin (AF) or arabinogalactan (A) on the tissue distribution of FA at 2 h after intravenous injection (12 mg/kg) to rats. AF or A was coinjected to rats with FA at a dose of 240 mg/kg. Values are given as mean ± S.D. for groups of three rats.

were nonlinear on a semilogarithmic scale as shown in Fig. 7. Apparent biological half lives estimated from the initial stage of the elimination curves were 2.5 min (3 mg/kg), 3.0 min (6 mg/kg), 5.5 min (12 mg/kg), 5.8 min (24 mg/kg), and 6.9 min (48 mg/kg), respectively.

After the final blood sampling at 2 h, the liver was excised and the level of FA (X_h) was determined. The hepatic uptake clearance (CL_h) was calculated by the Eq. (1). Fig. 8 shows a saturable distribution of FA into the liver. The hepatic uptake clearance was markedly reduced with an increase in dose due to the saturation in the hepatic uptake.

3.7. Receptor-mediated hepatic uptake of FA

An appreciable influence of asialofetuin or arabinogalactan on the plasma level of FA was seen in Fig. 9. The elimination of FA from the blood circulation was markedly retarded by the coadministration of asialofetuin (240 mg/kg). The same inhibition was also seen in arabinogalactan coadministration (Fig. 9).

The hepatic accumulation of FA was markedly reduced by both asialofetuin and arabinogalactan (Fig. 10). However, the renal accumulation of FA was not altered by asialofetuin or accelerated by arabinogalactan coadministration.

Hepatocellular localization of FA at 2 h after injection at a dose of 12 mg/kg was examined (Fig. 11). Results were compared with that of FITC-labeled dextran (FD-70) at 4 h after injec-

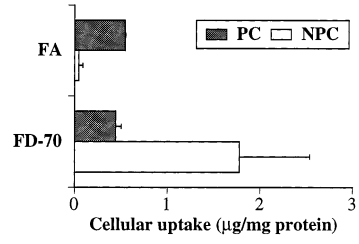

Fig. 11. Distribution of FA and FITC-labeled dextran (T-70) in parenchymal (PC) and nonparenchymal (NPC) cells after intravenous injection to rats at a dose of 12 mg/kg. Liver samples were collected at 2 h for FA or at 4 h for FD-70 after the drug administration. Values are given as mean ± S.D. for groups of three rats.

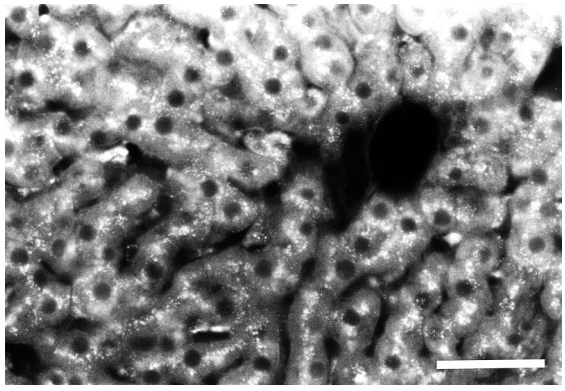

Fig. 12. Fluorescence microscopic examination of paraffin section of the rat liver at 1 day after intravenous injection of FA (120 mg/kg). The bar represents the length of 50 μm.

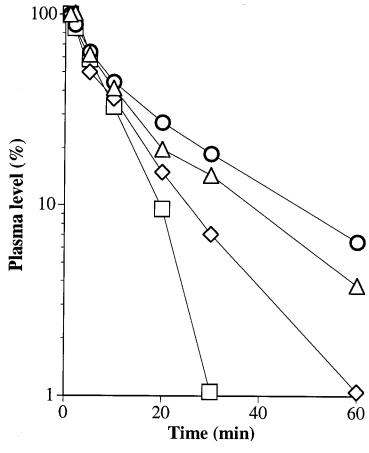

Fig. 13. Effects of chemically modified arabinogalactan on the plasma clearance of FA after intravenous injection (12 mg/kg) to rats. □, FA; ◇, FA and 25% $NaIO_4$-oxidized arabinogalactan; △, FA and 12.5% $NaIO_4$-oxidized arabinogalactan; ○, FA and arabinogalactan. Arabinogalactan or a chemically modified arabinogalactan was coinjected to rats with FA at a dose of 240 mg/kg.

tion at the same dose. FA was distributed mainly into the parenchymal cells, whereas FD-70 was predominantly taken up by the nonparenchymal cells.

Fluorescence microscopic examination of paraffin section of the liver revealed FA that was effectively endocytosed by the liver parenchymal cells (Fig. 12).

3.8. Evaluation of $NaIO_4$-oxidized arabinogalactan as a receptor-mediated drug carrier

Hepatic targetability of chemically modified arabinogalactans was examined with an in vivo animal experiment. Fig. 13 demonstrates the effects of coadministration of $NaIO_4$-oxidized arabinogalactans on the plasma clearance of FA after intravenous injection (12 mg/kg) to rats. The intact arabinogalactan markedly reduced the clearance of FA from the blood circulation. Oxidized arabinogalactan (12.5%) decreased the elimination rate of FA as well as the intact one, whereas the effect of 25% oxidized arabinogalactan was marginal (Fig. 13). Neither 100% oxidized nor 50% oxidized arabinogalactan affected the plasma clearance of FA (data are not shown).

Fig. 14 shows the reduced hepatic uptake of FA with coadministration of the chemically modified arabinogalactans, which fairly corresponded with the reduced clearance of FA from the blood circulation shown in Fig. 13. Oxidized arabinogalactan (12.5%) decreased the hepatic level of FA as well as the intact one (Fig. 14).

In order to confirm the hepatic targetability of chemically modified arabinogalactans, FITC-labeled $NaIO_4$-oxidized arabinogalactan was prepared. As shown in Fig. 15, the elimination of the FITC-labeled 12.5% oxidized arabinogalactan from the plasma was comparable to that of FA; the hepatic level of the former was 21% of dose which was identical to that of the latter (25% of dose).

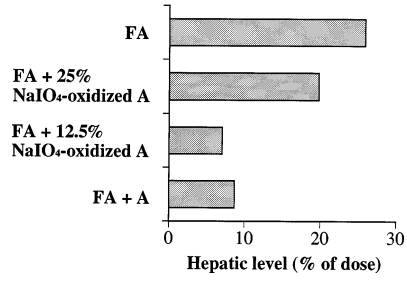

Fig. 14. Effects of chemically modified arabinogalactan on the hepatic level of FA at 2 h after intravenous injection (12 mg/kg) to rats. Arabinogalactan (A) or a chemically modified arabinogalactan was coinjected to rats with FA at a dose of 240 mg/kg.

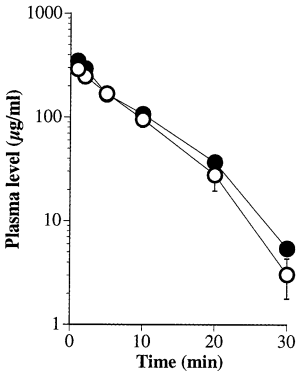

Fig. 15. Plasma level of FITC-labeled arabinogalactan after intravenous injection (12 mg/kg) to rats. ○, FA; ●, FITC-labeled 12.5% $NaIO_4$-oxidized arabinogalactan. Values are given as mean ± S.D. for groups of three rats.

4. Discussion

A high-performance size-exclusion chromatographic procedure for the quantitative analysis of FITC-labeled dextrans in biological media was first developed by Kurtzhals et al. (1989). Mehvar and Shepard (1992) have reported a systematic kinetic analysis of various molecular weights of FITC-labeled dextrans in rats by the HPSEC method.

We have successfully synthesized a FITC-labeled arabinogalactan according to the method of deBelder and Granath (1973). Then, a specific and sensitive HPSEC method has been established to measure the concentrations of arabinogalactan in serial tissues and urine samples. In this system, a good linear relationship was observed between the retention time of FITC-labeled dextrans and the logarithm of their molecular weights in the range of 4.4–144 kDa (data are not shown). Therefore, as shown in Fig. 6, the system can be also used to determine the molecular weight change of FA in the body based on the retention time alteration. The molecular weight of FA estimated by HPSEC was 12.6 kDa which was much smaller than that of 39.7 kDa by HPSEC-LALLS. This indicated that arabinogalactan has a highly branched structure, whereas dextrans are linear polysaccharides with some branching.

Although FA was basically stable in the body, some lower shift in the molecular weight distribution was seen in the kidney (Fig. 6). Since the size of FA (12.8 kDa) estimated by HPSEC was much smaller than the molecular weight threshold (45 kDa) limiting the glomerular filtration, a large portion of FA was rapidly eliminated in the urine within the first 6 h (Fig. 5). Therefore, FA was detected almost as an intact form in the urine in which the renal contribution to the degradation product was not estimated. On the other hand, the marked fragmentation of FA was demonstrated in the homogenate both of feces and large intestinal mucosa; the retention time of the product in the HPSEC analysis was 14.7 min (Kaneo, unpublished data).

A marked dose-dependency was found in the hepatic uptake of FA in rats (Fig. 8). Half maximum uptake was seen at the dose of about 12 mg/kg The amounts of FA recovered in the liver at 2 h after administration were 52.9, 39.4, 25.9, 20.9 and 14.0% of dose at doses of 3, 6, 12, 24 and 48 mg/kg, respectively. The blood persistence of FA increased with an increase in dose due to the saturation in the hepatic uptake (Fig. 7).

It was reported that serum glycoproteins bearing N-linked oligosaccharides after removal of terminal sialic acid residues are rapidly taken up by parenchymal liver cells (Ashwell and Morell, 1974). Hepatic recognition of asialoglycoproteins such as asialofetuin is dependent on the galactose residues which become exposed after desialylation and bind to an asialoglycoprotein receptor which is present in large amounts only on parenchymal liver cells.

The elimination of FA from the blood circulation was markedly decreased by the coadministration of asialofetuin (Fig. 9). In this case, both the total clearance (270 ml/h per kg body weight) and hepatic uptake clearance of FA (47.1 ml/h/kg body weight) were decreased to 119 and 4.53 ml/h per kg body weight, respectively. As shown in Fig. 11, FA was distributed mainly into the parenchymal liver cell fraction. Furthermore, the microscopic examination showed that FA was effectively endocytosed by parenchymal liver cells (Fig. 12). It is well established that asialoglycoproteins enter the cell via receptor-mediated endocytosis and finally end up in lysosomes. Distinct fluorescent vesicles were appeared in the hepato-

cyte within 30 min after intravenous injection of FA and could be observed for at least 1 week, suggesting that FA might be accumulated in lisosome fraction via endosome particulates. These results suggested that FA has a high affinity for the asialoglycoprotein receptor and the hepatic uptake of FA proceeds via receptor-mediated endocytosis.

As shown in Fig. 2, FA was mainly distributed in both the liver and the kidney. The distribution of FA in the liver was markedly inhibited by the coadministration of asialofetuin, whereas the competitive inhibition was not seen in the renal distribution of FA (Fig. 10). The asialoglycoprotein receptor is predominantly present on parenchymal liver cells whereas small amounts of this receptor were also detected in several organs of gastrointestinal apparatus, spleen and kidney. In these extrahepatic tissues, however, receptor binding capacity was found to be only 1–5% of that of liver asialoglycoprotein receptor (Mu et al., 1993). The renal uptake of FA seen in rats was not observed in mice, suggesting the existence of species difference (Kaneo, unpublished data).

Arabinogalactan is a naturally occurring polysaccharides which has an advantage over the other hepatotropic carriers since it does not require chemical changes in order to bind to the asialoglycoprotein receptor. However, it must be chemically modified in order to acquire functional groups to which guest molecules can be bound. A periodate activation method has been applied to polysaccharides such as cellulose, starch and dextran (Campbell, 1963; Bernstein et al., 1978; Singh et al., 1982; Wileman et al., 1983; Larsen, 1989). The polyaldehydes were subsequently coupled with amino-type drugs either via Schiff-base formation or, if performed in the presence of a reducing agent such as sodium borohydride or sodium cyanoborohydride, via alkylamine formation.

The aldehyde content of the $NaIO_4$-oxidized arabinogalactan was determined by the Romijin method. The numbers of aldehyde groups per sugar unit (CHO mol/sugar mol) for the oxidized arabinogalactans were 1.05 (100% oxidized), 0.607 (50% oxidized), 0.344 (25% oxidized) and 0.182 (12.5% oxidized), respectively. Arabinogalactan oxidized more than 25% had perfectly lost the inhibitory effect on the hepatic uptake of FA (Figs. 13 and 14). It was found that the destruction of sugar structure of terminal galactose caused elimination of the specific recognition of arabinogalactan for the asialoglycoprotein receptor. Among the partially oxidized arabinogalactans, 12.5% oxidized one kept a good hepatic targeting ability as shown in Fig. 15. Experimental studies on conjugation of guest molecules such as albumin, lysozyme, trypsin, etc. to the 12.5% oxidized arabinogalactan showed the feasibility of hepatotropic targeting system with arabinogalactan as a carrier (Kaneo, unpublished data).

5. Conclusion

Our results demonstrate that arabinogalactan can serve as a potential carrier for the delivery of enzymes and drugs to the liver parenchymal cells via the asialoglycoprotein receptor. The data presented here may be used in the future design of drug delivery studies with arabinogalactan.

Acknowledgements

Technical assistance by Kazuto Higashi, Reiko Tamaki, Junko Taguchi, and Susumu Yamaguchi is gratefully acknowledged. The authors are also grateful to Mr. Masuo Umino of Tosoh Co., Ltd. for the gift of TSK gel G3000SWXL columns and the molecular weight determination of arabinogalactan by HPSEC-LALLS.

References

Adams, M.K., Douglas, C., 1963. Arabinogalactan — a review of the literature. Tech. Assoc. Pulp Paper Ind. 46, 544–548.

Ashwell, G., Morell, A.G., 1974. The role of surface carbohydrates in the hepatic recognition and transport of circulating glycoproteins. Advan. Enzymol. 41, 99–128.

Bernstein, A., Hurwitz, E., Maron, R., Arnon, R., Sela, M., Wilechek, M., 1978. Higher antitumor efficacy of daunomycin when linked to dextran: in vivo and in vitro studies. J. Natl. Cancer Inst. 60, 379–384.

Campbell, L.A., 1963. Pharmaceutical aspects of a *p*-aminosalicylate dialdehyde starch compound. J. Pharm. Sci. 52, 76–78.

Cui, L., Faraj, A., E1Alaoui, A.M., Groman, E.V., Rutkowski, J.V., Josephson, L., Sommadossi, J.-P., 1997. Arabinogalactan (9 KDa)-9β-D-arabinofuranosyladenine-5′-monophosphate, a novel liver-targeted conjugate that selectively inhibits hepatitis B virus replication in vitro. Antivir. Chem. Chemother. 8, 529–536.

deBelder, A.N., Granath, K., 1973. Preparation and properties of fluorescein-labelled dextrans. Carbohydr. Res. 30, 375–378.

DiStefano, G., Fiume, L., 1997. Liver glycotargeting of antiviral nucleoside analogues. Trends Glycosci. Glycotech. 9, 461–472.

Enriquez, P.M., Jung, C., Josephson, L., 1995. Conjugation of adenine arabinoside 5′-monophosphate to arabinogalctan: synthesis, characterization, and antiviral activity. Bioconjugate Chem. 6, 195–202.

Groman, E.V., Enriquez, P.M., Jung, C., Josephson, L., 1994. Arabinogalactan for hepatic drug delivery. Bioconjugate Chem. 5, 547–556.

Josephson, L., Groman, E.V., Menz, E., Lewis, L.M., Bengele, H., 1990. A functionalized superparamagnetic iron oxide colloid as a receptor directed MR contrast agent. Magn. Reson. Imaging 8, 637–646.

Kurtzhals, R., Larsen, C., Johansen, M., 1989. High-performance size-exclusion chromatographic procedure for the determination of fluoresceinyl isothiocyanate dextrans of various molecular masses in biological media. J. Chromatogr. 491, 117–127.

Larsen, C., 1989. Dextran prodrugs-structure and stability in relation to therapeutic activity. Adv. Drug Deliv. Rev. 3, 103–154.

Mehvar, R., Shepard, T.L., 1992. Molecular-weight-dependent pharmacokinetics of fluorescein-labeled dextrans in rats. J. Pharm. Sci 81, 908–912.

Meijer, D.K.F., Jansen, R.W., Molema, G., 1992. Drug targeting systems for antiviral agents: options and limitations. Antivir. Res. 18, 215–258.

Monsigny, M., Roche, A., Midoux, P., Mayer, R., 1994. Glycoconjugates as carriers for specific delivery of therapeutic drugs and genes. Adv. Drug Deliv. Rev. 14, 1–24.

Mu, J.-Z., Tang, L.-H., Alpers, D.H., 1993. Asialoglycoprotein receptor mRNAs are expressed in most extrahepatic rat tissues during development. Am. J. Physiol. 264, G752–762.

Naim, J.O., vanOss, C.J., 1992. The effect of hydrophilicity-hydrophobicity and solubility on the immunogenicity of some natural and synthetic polymers. Immunol. Invest. 21, 649–662.

Odonmazig, P., Ebringerova, A., Machova, E., Alfoldi, J., 1994. Structural and molecular properties of the arabinogalactan isolated from Mongolian larchwood. Carbohydr. Res. 252, 317–324.

Seglen, P.O., 1976. Preparation of isolated liver cells. Methods Cell Biol. 13, 29–83.

Singh, M., Ray, A.R., Vasudevan, P., 1982. Biodegradation studies on periodate oxidized cellulose. Biomaterial 3, 16–20.

Takakura, Y., Fujita, T., Hashida, M., Sezaki, H., 1990. Disposition characteristics of macromolecules in tumor-bearing mice. Pharm. Res. 7, 339–346.

Uhlenbruck, G., Beuth, J., Oette, K., Ko, H.L., Pulverer, G., 1987. Prevention of experimental liver metastases by D-galactose. Experientia 43, 437–438.

Wadhwa, M.S., Rice, K.G., 1995. Receptor mediated glycotargeting. J. Drug Target. 3, 111–127.

Weissleder, R., Reimer, P., Lee, A.S., Wittenberg, J., Brady, T.J., 1990. MR receptor imaging: ultrasmall iron oxide particles targeted to asialoglycoprotein receptors. Am. J. Roentgenol. 155, 1161–1167.

Wileman, T., Bennett, M., Lilleymann, J., 1983. Potential use of an asparaginase-dextran conjugate in acute lymphoblastic leukaemia. J. Pharm. Pharmacol. 35, 762–765.

Yamaoka, K., Tanigawara, Y., Nakagawa, T., Uno, T., 1981. A pharmacokinetic analysis program (MULTI) for microcomputer. J. Pharm. Dyn. 4, 879–885.