Macromolecule–Macromolecule Interaction in Drug Distribution: Effect of α-Globulin Concentration on the Hepatic Uptake of Fractionated ^{3}H-Heparin by Perfused Rat Liver

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The effect of α-globulin, the dominant binding protein for fractionated ^{3}H-heparin, on the hepatic uptake of ^{3}H-heparin was studied by liver perfusion experiments in rats. Fractionated ^{3}H-heparin concentration in the recirculated perfusate declined one-exponentially with time for each of six initial concentration levels of α-globulin. The hepatic uptake clearance of fractionated ^{3}H-heparin was 0.154 ml/min/g liver in the absence of α-globulin, and it decreased with increasing α-globulin concentrations. This result indicates that the hepatic uptake rate of α-globulin-bound fractionated ^{3}H-heparin is lower than that of unbound fractionated ^{3}H-heparin.

On the other hand, it was indicated that almost all fractionated ^{3}H-heparin binds to α-globulin at 8 mg/ml of α-globulin in *in vitro* study. However, the hepatic uptake clearance of the heparin at the concentration was of a certain value that could not to be ignored. It was suggested that α-globulin-bound fractionated ^{3}H-heparin also contributed to the hepatic uptake of fractionated ^{3}H-heparin. Therefore, a protein-mediated transport system, which has been reported for some low molecular weight drugs, may also exist in the hepatic uptake of such a high molecular weight compound as fractionated ^{3}H-heparin.

Keywords rat liver perfusion; hepatic uptake; fractionated ^{3}H-heparin; α-globulin; protein binding; macromolecule–macromolecule interaction; protein-mediated transport

Heparin is a water soluble and acidic mucopolysaccharide, and has been frequently used for prophylaxis of thrombosis such as myocardial infarction. However, the pharmacokinetic features of the drug have not yet been clarified, because commercially available heparin is polydisperse with respect to molecular weight and heterogeneous with respect to biological and chemical properties. Therefore, it was considered necessary to fractionate the commercially available heparin to diminish this polydispersity and heterogeneity before its use in a pharmacokinetic study.

In our laboratory, commercial ^{3}H-heparin was fractionated first by affinity chromatography on protamine-Sepharose in regard to the heterogeneity, and secondly by gel chromatography on Sephadex G-100 in regard to the polydispersity. Thus obtained, fractionated ^{3}H-heparin has been employed for pharmacokinetic studies in rats.[1–6] These investigations demonstrated that fractionated ^{3}H-heparin is mainly taken up by the liver after intravenous injection to rats.[2–6] On the other hand, a previous study[7] on plasma protein binding of fractionated ^{3}H-heparin clarified that α-globulin, rather than albumin, is the dominant binding protein for fractionated ^{3}H-heparin, although albumin is known as the dominant binding protein for a number of low molecular drugs.

In this study, the effect of α-globulin on the hepatic uptake of fractionated ^{3}H-heparin was investigated by liver perfusion experiments in rats.

Experimental

Materials [^{3}H(G)]Heparin sodium salt of porcine mucosal origin (# 2275-214: specific radioactivity was 11.1 MBq/mg: M.W. 6000—20000 daltons (Da) was purchased from NEN Research Products (Boston, Mass., U.S.A.), and bovine α-globulin (Cohn fraction IV-1) from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). The scintillation media for radioactivity measurements were Scintisol EX-H (Dojindo Laboratories, Kumamoto, Japan) and Biofluor (NEN Research Products) in the fractionation and liver perfusion experiment, respectively. All other chemicals were of analytical grade and used without further purification.

Fractionation of ^{3}H-Heparin Commercially available ^{3}H-heparin was fractionated by affinity chromatography on protamine-Sepharose as described in the preceding paper.[2] Subsequently, the fraction which showed peak affinity was fractionated by gel filtration chromatography on a Sephadex G-100 as described in the preceding paper.[3] In this study, fractionated ^{3}H-heparin of 15000—19500 Da was used for the liver perfusion experiments.

Preparation of Perfusate For preparation of a perfusate containing 10% plasma, male Wistar rats, 18 weeks old (350—400 g), were used to obtain the plasma. The rats were anesthetized with urethane. Blood samples were withdrawn through polyethylene tubing PE-50 (Clay Adams, Parssippany, N. J., U.S.A.) from the jugular artery, transferred to a centrifugation tube in which a citric acid solution (3.8%) was added beforehand in the proportion of one part citric acid solution to nine parts blood, and then centrifuged at 4 °C and 3000 rpm for 15 min.

To prepare a perfusate containing 0.0294 μM of fractionated ^{3}H-heparin, the fractionated ^{3}H-heparin solution was diluted with a pH 7.4 buffer solution (121 mM NaCl, 6 mM KCl, 0.74 mM KH_2PO_4, 0.6 mM $MgSO_4$, 12 mM $NaHCO_3$, 5 mM glucose), and α-globuin or rat plasma was added.

Perfusion of Rat Liver Male Wistar rats, 6 weeks old (175—220 g) were used without fasting throughout the experiments. After anesthetizing each rat with urethane (1000 mg/kg, i.p.), the liver was isolated by the method of Iwamoto *et al.*[8,9] A Miller-type organ perfusion apparatus[10] equipped with a peristaltic pump was used for the *in vitro* liver recirculation experiments at 37 °C. The volume of perfusate was 100 ml and the flow rate was fixed at 30 ml/min. One hundred microliters of perfusate were drawn at the programmed time for 60 min and the bile samples were collected at 10-min intervals. The radioactivity of these two samples was measured. After 60-min perfusion, the whole liver was homogenized with Polytron (PT20″OD″S, KINEMATICA GmbH, Switzerland). Tissue solubilizer (Protosol, NEN Research Products) was added to part of the homogenate, and then radioactivity was measured. As a measure of viability of the liver, glutamic-oxaloacetic transaminase (GOT) activity[11] was determined. Two hundred microliters of perfusate were taken for the GOT activity measurement at 5, 10, 20, 30, 40, 50 and 60 min after the start of perfusion. Only data of which GOT activities were less than 40 Kermen unit/ml during the experiment were adopted for analysis in the perfusion study.

Calculations Under the condition that only unbound fractionated ^{3}H-heparin is extracted from blood flow by the liver, the following equation is derived if the intrinsic uptake clearance is sufficiently small in comparison to the blood flow,

$$CL_H = f \times CL_{int} \quad (1)$$

where CL_H is the apparent hepatic uptake clearance, f is the fraction unbound and CL_{int} represents intrinsic uptake clearance. In the binding study, the fraction unbound, f, is given by the following equation:

$$f=\frac{K_d+C_f}{K_d+n\times P_t+C_f} \quad (2)$$

where K_d is the dissociation constant, C_f is the concentration of unbound fractionated ^{3}H-heparin, n is the number of binding sites on the surface of protein, and P_t[12] is the concentration of α-globulin. Although the K_d value is unknown for the fractionated ^{3}H-heparin, K_d values for commercial heparin are reported as 0.035—1.0 μM.[13-16] The concentration of fractionated ^{3}H-heparin (C_t) was 0.029 μM in this study. Therefore, it is assumed that K_d is larger than C_t in the liver perfusion experiment. On this assumption, Eq. 2 produces the following equation:

$$f=\frac{K_{d,PERF}}{K_{d,PERF}+n\times P_t}=\frac{K_{d,PERF}/n}{K_{d,PERF}/n+P_t} \quad (3)$$

where $K_{d,PERF}$ is the dissociation constant in the liver perfusion experiment. The following equation is then derived from Eq. 1 and 3:

$$CL_H=CL_{int}\times\frac{K_{d,PERF}/n}{K_{d,PERF}/n+P_t} \quad (4)$$

Substituting $K_{d,PERF}/n$ for K, Eq. 4 becomes Eq. 5:

$$CL_H=CL_{int}\times\frac{K}{K+P_t} \quad (5)$$

Equation 5 was used for the analysis of the relationship between the hepatic uptake of fractionated ^{3}H-heparin and the α-globulin concentration.

Results

Recovery of Radioactivity Recoveries of radioactivity from the perfusate and liver are shown in Table I. The percentage of recovery from the liver decreased with an increase in α-globulin concentration. Total recoveries are about 81—94%, which gives an approximate profile of elimination from the perfusate. Excretions into bile are small enough to be ignored (Table I).

Time Course of Fractionated ^{3}H-Heparin Remaining in the Perfusate The fractionated ^{3}H-heparin in the perfusate was eliminated by first-order kinetics regardless of existence of α-globulin, as shown in Fig. 1. The elimination of fractionated ^{3}H-heparin from the perfusate containing 1.0 mg/ml α-globulin was slower than that from the perfusate without α-globulin. The fractionated ^{3}H-heparin concentrations also decreased mono-exponentially with other α-globulin concentrations. These elimination curves were analyzed by fitting the one-compartment model equation: $C=C_0\cdot\exp(-k_{el}\cdot t)$ by the least squares regression program, MULTI.[18] The results are shown in Table II.

The elimination rate constant (k_{el}) for the fractionated

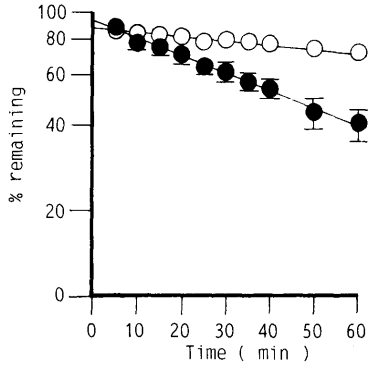

Fig. 1. Time Course of Radioactivity of Fractionated ^{3}H-Heparin Remaining in the Perfusate from Liver Perfusion Experiments

○, 1.0 mg/ml α-globulin; ●, in the absence of α-globulin. Each point and vertical bar represents the mean ± S.D. from three rats. The solid lines represent the results of fitting by a least squares regression program, MULTI.[18]

TABLE I. Recovery of Radioactivity at 60 min in Liver Recirculation Experiments

α-Globulin conc. (mg/ml)	Percent of initial load[a]						
	0	0.25	0.5	1.0	2.0	4.0	8.0
Perfusate	40.6±5.3	58.5±8.3	64.3±5.5	72.0±1.2	76.3±3.9	75.4±4.8	65.8±3.7
Liver	40.3±2.1	27.4±6.3	23.4±6.4	21.7±3.0	17.4±3.9	16.0±2.7	18.3±6.4
Bile[b]	0.026±0.033	0.100±0.132	0.031±0.042	0.003±0.003	0.025±0.016	0.003±0.004	0.069±0.033
Total	81.0±4.0	86.0±3.7	87.7±6.6	93.7±1.9	93.7±1.2	91.4±3.1	84.1±2.8

Each value represents the mean ± S.D. from three rats. *a*) Initial load was 2.94 μM (0.555 MBq). *b*) Cumulative excretion.

TABLE II. Pharmacokinetic Parameters from Liver Recirculation Experiments with Varying Concentrations of α-Globulin in the Perfusate

	Liver weight (g)	k_{el} (10^{-2} min^{-1})	V_d (ml)	CL_H[a] (ml/min/g liver)	f[b]
α-Globulin (mg/ml)					
0	9.82±0.61	1.39±0.12	108.3±5.7	0.154±0.013[c]	—
0.25	9.56±0.94	0.54±0.23	128.0±4.2	0.071±0.026	0.463
0.5	8.98±0.69	0.44±0.19	121.7±4.7	0.061±0.029	0.397
1.0	8.82±0.47	0.32±0.05	114.3±3.7	0.042±0.004	0.271
2.0	9.44±0.50	0.35±0.14	111.4±4.4	0.035±0.015	0.225
4.0	10.60±1.78	0.18±0.09	118.1±1.3	0.020±0.010	0.128
8.0	10.52±0.41	0.33±0.07	123.4±4.8	0.039±0.009	0.251
10% plasma[d]	8.94	0.51	111.4	0.063	0.410
In vivo	—	—	—	0.020	—

Each value represents the mean ± S.D. of three rats, unless otherwise indicated.

a) CL_H: hepatic uptake clearance, $CL_H=\frac{k_{el}\cdot V_d}{\text{liver weight}}$. *b*) $f=\frac{CL_H}{CL_{int}}$, CL_{int}: intrinsic hepatic uptake clearance. *c*) Observed CL_{int}. *d*) $n=1$.

^{3}H-heparin decreased as the α-globulin concentration in the perfusate increased. The distribution volume (V_d), calculated from the initial load and the initial concentration (C_0), took the values of 108.3±5.7—128.0±4.2 ml as shown in Table II. These V_d values are greater than the volume of perfusate (100 ml) and cannot be explained by dead volume in the recirculation apparatus (0.26 ml), vascular space in the liver (0.7 ml) or Disse's space (0.84 ml).[17] Therefore, it is suggested that fractionated ^{3}H-heparin is rapidly taken up into hepatocytes or is quickly adsorbed to hepatocytes just after the start of the perfusion experiments.

The apparent hepatic uptake clearances (CL_H) were calculated using the k_{el} and V_d values which are also shown in Table II. The flow rate of perfusate was about 3 ml/min/g liver. This was much higher than the apparent hepatic uptake clearance for fractionated ^{3}H-heparin. Therefore, this hepatic uptake is not limited by the flow rate but by the intrinsic uptake clearance of the liver.

Effect of α-Globulin Concentration on Hepatic Uptake of Fractionated ^{3}H-Heparin The apparent hepatic clearance, CL_H value, decreased with an increase in α-globulin concentration (Table II). The decrease in CL_H suggests that the uptake of protein-bound fractionated ^{3}H-heparin is slower than that of unbound ^{3}H-heparin. The intrinsic hepatic uptake clearance (CL_{int}) was estimated from the experiment in the absence of α-globulin. The value is 0.154 ml/min/g liver. Assuming that only unbound drugs are extracted by the liver, the apparent fraction unbound, f, is calculated from Eq. 1 as indicated in the footnote in Table II. The estimated f values are shown on the right-hand side of the table.

To clarify the uptake mechanism of the recirculated rat liver, the data for the apparent hepatic clearance (CL_H) and the α-globulin concentration (P_t) were fitted to the Eq. 5 by a least squares regression program, MULTI.[18] The estimated parameters for CL_{int} and K are 0.15 ml/min/g liver and 0.31 μM, respectively. The calculated CL_{int} is in excellent agreement with the observed CL_{int} in Table II (footnote c). The solid line in Fig. 2 represents the best fit curve, which agrees well with the experimental data. The result of the experiment can be elucidated by Eq. 5.

When the perfusate containing 10% plasma (almost equivalent to α-globulin concentration: 0.8—1.2 mg/ml) was used, the CL_H took the value of 0.063 ml/min/g liver, which was greater than the CL_H value at 1.0 mg/ml α-globulin, but considerably less than that without α-globulin.

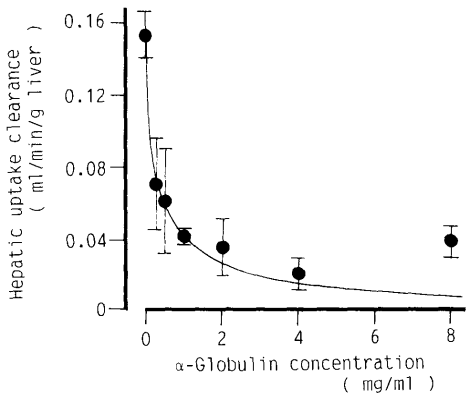

Fig. 2. Hepatic Uptake Clearance for Fractionated ^{3}H-Heparin *versus* α-Globulin Concentration

Each point and vertical bar represents the mean±S.D. of three experiments. The solid line represents the result of fitting, using Eq. 5.

Discussion

In the previous study, it was demonstrated that α-globulin is the dominant binding protein of fractionated ^{3}H-heparin in plasma.[7] In this study, the hepatic uptake of the fractionated ^{3}H-heparin decreased with an increase in α-globulin concentration in the perfusate. This result suggests that the uptake of bound fractionated ^{3}H-heparin is slower than that of unbound ^{3}H-heparin. Besides, the observed data for the apparent hepatic clearance (CL_H) showed close agreement with Eq. 5. The equation was derived from the assumption that the fractionated ^{3}H-heparin is taken up into the liver only in an form unbound to α-globulin in the perfusate.

Meanwhile, the result of gel filtration chromatography suggests that fractionated ^{3}H-heparin is nearly 100% bound to α-globulin at the normal blood concentration of α-globulin (8—12 mg/ml),[7] and the fractionated ^{3}H-heparin is taken up significantly by the liver, even at 8 mg/ml α-globulin in this study, which gives an apparent f value of 0.251 instead of zero (Table II, Fig. 2). These results show that hepatic uptake at 8 mg/ml α-globulin is much more rapid than that presumed by *in vitro* binding data. Recently, similar phenomena have been reported in regard to some low molecular drugs, and a protein-mediated transport hypothesis has been proposed.[19-26] This study is the first one in which protein-mediated transport is reported in regard to a high molecular drug.

There are two hypothetical models that elucidate protein-mediated transport of low molecular drugs. One is the "albumin receptor model,"[19-22] where ligand–albumin complex binds to a specific receptor on the surface of cell and the ligand is taken up at a higher rate than the intrinsic clearance. In this case, the binding parameters obtained by *in vivo* experiments are similar to those by *in vitro* experiments, and the actual existence of a saturable binding site for albumin on hepatic cells has been demonstrated. However, a similar phenomenon has not yet been found for α-globulin. Another hypothetical model is the so-called "enhanced dissociation model,"[23-26] where ligand–plasma protein complex passes through the blood vessels of various systems, a conformation change of the complex occurs as the result of an interaction with the surface of the cell, and the concentration of unbound ligand increases. In this case, the apparent uptake clearances for protein–ligand complex never exceed the intrinsic clearance for unbound ligand. While a number of protein-mediated transport phenomena have been reported, conclusive evidence has not been obtained.

In conclusion, our experimental data indicate that fractionated ^{3}H-heparin bound to α-globulin may be taken up by the liver, though the uptake rate is slower than that of unbound ^{3}H-heparin. The result suggests the involvement of "protein-mediated transport" in the hepatic uptake of fractionated ^{3}H-heparin. However, our data is not detailed enough to determine the uptake mechanism. Experiments are now in progress in our laboratory to investigate the kinetics and mechanisms of fractionated ^{3}H-heparin transport to the liver.

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