ELSEVIER

International Journal of Pharmaceutics 151 (1997) 15–26

international journal of pharmaceutics

Pharmacokinetic analysis of scavenger receptor-mediated uptake of anionized proteins in the isolated perfused rat liver

Hisao Furitsu, Ken-ichi Ogawara, Takuya Fujita [1], Fumiyoshi Yamashita, Yoshinobu Takakura, Hitoshi Sezaki [2], Mitsuru Hashida *

Department of Drug Delivery Research, Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-01, Japan

Received 1 October 1996; revised 26 December 1996; accepted 13 January 1997

Abstract

The hepatic uptake characteristics of ^{111}In-labeled succinylated lysozyme (Suc-LZM), superoxide dismutase (Suc-SOD), bovine serum albumin (Suc-BSA), catalase (Suc-CAT), and maleylated SOD (Mal-SOD), BSA (Mal-BSA) were studied in rat liver perfusion experiments. During a single-pass constant infusion mode, [^{111}In]Suc-BSA, [^{111}In]Suc-CAT, and [^{111}In]Mal-BSA were significantly extracted while extraction of [^{111}In]Suc-LZM, [^{111}In]Suc-SOD, and [^{111}In]Mal-SOD were small, suggesting the importance of molecular weight or total number of anionic charges per one protein molecule for the hepatic uptake of anionized proteins. The extraction ratio at steady state (E_{ss}) for [^{111}In]Suc-BSA was significantly decreased by co-administration of Mal-BSA or dextran sulfate, which is known to be taken up via scavenger receptor, and NH_4Cl, dinitrophenol, or cytochalasin B, suggesting that hepatic uptake of [^{111}In]Suc-BSA proceeds via receptor-mediated endocytosis. The internalization rate constant (k_{int}) for [^{111}In]Suc-BSA was calculated to be 0.27 min^{-1} in liver perfusion experiments using the acid-wash method. The outflow patterns of [^{111}In]Suc-BSA at various inflow concentrations were simultaneously fitted to a physiological one-organ pharmacokinetic model, in which the hepatic uptake was represented by division into the processes of binding to the cell surface and internalization, by the use of the MULTI (RUNGE) program. The obtained pharmacokinetic parameters (maximum binding amount X_∞, binding constant K, and internalization rate constant k_{int}) for [^{111}In]Suc-BSA clearly characterized the difference in their hepatic uptake mechanisms compared with lactosylated and cationized BSA. The present study has demonstrated that large succinylated and maleylated proteins should be useful as a carrier for the intracellular delivery of drugs specifically into the liver endothelial cells. © 1997 Elsevier Science B.V.

Keywords: Succinylated proteins; Rat liver perfusion; Constant infusion; Pharmacokinetic analysis; Binding; Internalization; Model analysis

* Corresponding author.

[1] Present address: Department of Biopharmaceutics, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607, Japan.

[2] Present address: Faculty of Pharmaceutical Sciences, Setsunan University, Hirakata 573-01, Japan.

PII S0378-5173(97)04899-0

Table 1
Physicochemical properties of anionized proteins

Compound	Molecular weight[a]	Number of NH_2-group[b]	% Remaining enzymatic activity[c]	Isoelectric point[d]
Lysozyme	14 300	9.0	—	11.0
Suc-LZM	15 000	2.0	—	<4.0
SOD	32 000	24.0	100	5.0–5.2
Suc-SOD	34 000	4.3	55.2	<4.0
Mal-SOD	34 000	6.8	60.0	
BSA	67 000	60.0	—	4.7–4.9
Suc-BSA	71 000	20.0	—	<4.0
Mal-BSA	72 000	12.3	—	<4.0
Catalase	220 000	162.0	100	5.7
Suc-CAT	221 000	82.0	98.1	<4.0

[a] The molecular weights of compounds were estimated by HPLC gel-filtration chromatography using Shim-pack Diol-300 column (Shimadzu, Kyoto, Japan).
[b]The numbers of amino group were determined by trinitrobenzensulfonic acid method.
[c]SOD and catalase enzymatic activities were assayed by nitroblue tetrazolium reduction method and determination of H_2O_2 removal by spectrophotometry, respectively.
[d]Isoelectric points of compounds were confirmed by chromatofocusing using Polybuffer exchanger 94 resin and the Polybuffer 74 elution buffer system (Pharmacia, Uppsala, Sweden).

1. Introduction

In our series of investigations, we have systematically studied the pharmacokinetic properties of macromolecules in relation to their physicochemical characteristics such as molecular weight and electric charge using in vivo and in situ liver perfusion experiments (Takakura et al., 1987, 1990; Nishida et al., 1990, 1991). Based on the findings, we have developed a variety of targeted delivery systems for low molecular weight drugs and protein drugs (Fujita et al., 1992a,b; Nishikawa et al., 1993; Hirabayashi et al., 1996). In a previous paper, we have demonstrated that macromolecules having strong anionic charges, such as succinylated proteins and dextran sulfate, are taken up by the liver via scavenger receptor-mediated mechanism after intravenous injection in mice (Takakura et al., 1994). The study has suggested that hepatic targeting of protein drugs can be achieved by direct succinylation.

For further understanding of the hepatic uptake of anionized proteins by the scavenger receptors, we performed pharmacokinetic studies using a rat liver perfusion system in the present study. Hepatic disposition of various strongly anionized proteins including succinylated LZM, SOD, BSA, CAT and maleylated SOD, BSA was studied in the perfusion experiment. Furthermore, the binding and subsequent internalization of [^{111}In]Suc-BSA by the liver cells were evaluated with two kinds of methods, acid-wash technique and model analysis using a physiological one-organ pharmacokinetic model.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 190–210 g were used and were maintained on standard rat foods and water ad libitum.

2.2. Materials

BSA (crystallized, MW 67 000) was purchased from ICN Biomedical Costa Mesa, CA. Lysozyme (LZM, MW 14 000), catalase (CAT, MW 220 000), and dextran sulfate (DS, MW 8000) were from Sigma (St. Louis, MO). Recombinant human Cu/Zn superoxide dismutase (SOD, MW 32 000) was kindly supplied by Asahi, Shizuoka, Japan. Succinic anhydride and maleic

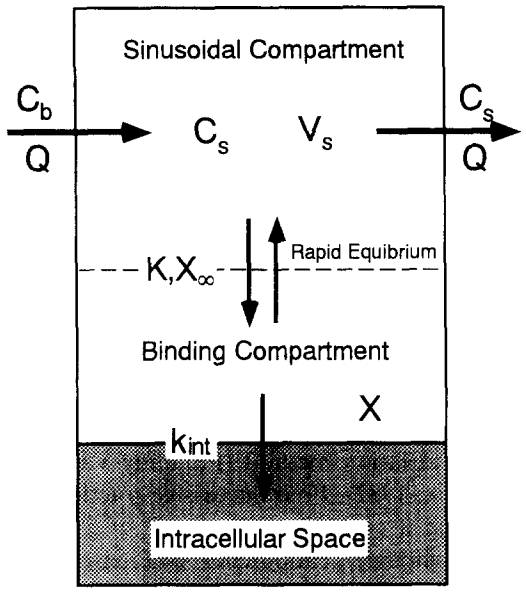

Fig. 1. Physiological pharmacokinetic model for hepatic uptake of anionized proteins. Q, flow rate (ml/min); C_b, inflow concentration (μg/ml); C_s, sinusoidal concentration (μg/ml); V_s, sinusoidal volume (ml); X, binding amount (μg); X_∞, maximum binding amount (μg); K, binding constant (ml/μg); k_{int}, internalization rate constant (min^{-1}).

anhydride were from Nacalai Tesque (Kyoto, Japan). ^{111}Indium chloride ([^{111}In]Cl_3) was kindly supplied by Nihon Medi-Physics (Takarazuka, Japan). All other chemicals were reagent grade products.

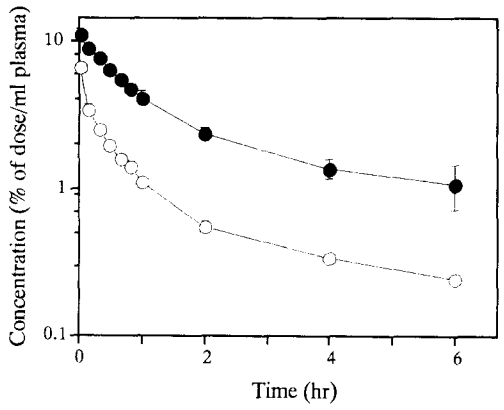

Fig. 2. Plasma concentration of [^{111}In]Suc-BSA after intravenous injection into rats at doses of 1 mg/kg (○) and 50 mg/kg (●). Values are means of three experiments, with the vertical bars indicating the S.D.

2.3. Synthesis and characterization of succinylated and maleylated proteins

Succinylated and maleylated proteins were synthesized as reported previously (Tokuda et al., 1993). In brief, for succinylated BSA (Suc-BSA), BSA (500 mg) was dissolved in 25 ml of 0.2 M Tris buffer (pH 8.65) and 120-fold molar succinic anhydride (90 mg) in 100 μl of $(Me)_2SO$ was added. The mixture was stirred for 1 h at room temperature. For maleylated BSA (Mal-BSA), BSA (100 mg) was reacted with an excess amount of recrystallized maleic anhydride in 10 ml of 0.1 M $Na_4P_2O_7$ (pH 9.0) and the mixture was stirred for 5 min at 4°C with maintaining the pH at 9.0. The modified BSA were purified by gel filtration and concentrated by ultrafiltration, and then lyophilized. Succinylated LZM (Suc-LZM), SOD (Suc-SOD), CAT (Suc-CAT) and maleylated SOD (Mal-SOD) were also synthesized by the same methods in smaller scales using less succinic anhydride to maintain their biological activities. The degree of modification was determined by measuring the amount of free amino groups in the modified BSA with trinitrobenzene sulfonate (Habeeb, 1966). Isoelectric points of proteins were determined by chromatofocusing method using Polybuffer exchanger 94 resin and the Polybuffer 74 elution buffer system (Pharmacia, Uppsala, Sweden). Enzymatic activities of SOD and CAT were assayed by nitroblue tetrazolium reduction method using a SOD test kit (Wako Pure Chemical, Osaka, Japan) and determination of H_2O_2 removal by spectrophotometry (Hugo, 1984), respectively. The physicochemical characteristics of these anionized proteins are summarized in Table 1.

2.4. Radiolabeling of succinylated or maleylated proteins

After succinylation or maleylation, proteins were radiolabeled with ^{111}In using the bifunctional chelating agent diethylenetriaminepentaacetic acid anhydride according to the method of Hnatowich

Table 2
Pharmacokinetic parameters for [^{111}In]Suc-BSA after intravenous injection into rats

Dose (mg/kg)	AUC (% dose h/ml)	Clearance (μl/h)		Tissue uptake rate index (μl/h/g)			
		CL_{total}	CL_{liver}	Liver	Spleen	Kidney	Heart
1	5.7	17 700	9222	1016	738	492	16.3
50	21.8	4580	1299	154	103	277	7.8

et al. (1982). This method was selected for accurate estimation of organ uptake of succinylated or maleylated proteins because the efflux of the radioactive metabolite of ^{111}In-labeled proteins from the cells was reported to be negligible (Duncan and Welch, 1993; Arano et al., 1994). The ^{111}In-labeled succinylated or maleylated proteins had a specific activity of $\approx$ 37 MBq/mg.

2.5. In vivo disposition experiment

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (20 mg/kg) and saline solutions of [^{111}In]Suc-BSA (1 or 50 mg/kg) were injected into a femoral vein. The body temperature of rats was kept at 37°C by a heat lamp during the experiment. Blood samples (0.2 ml) were withdrawn from the jugular vein over 6 h and centrifuged at 3000 rpm for 2 min. At 6 h after injection, the liver and the other organs were excised, rinsed with saline, and weighed, and their radioactivities were counted. The area under the plasma concentration-time curves (AUC) was calculated by the trapezoidal method (Yamaoka et al., 1978). Apparent total-body clearance (CL_{total}) and hepatic clearance (CL_{liver}) were calculated by dividing the injection dose and the total uptake amount in the liver by AUC, respectively. Tissue uptake rate index were defined as organ clearance normalized by the weight of the organ (Takakura et al., 1987).

2.6. Cellular distribution of succinylated BSA in the liver after intravenous injection

One h after intravenous injection of [^{111}In]Suc-BSA at a dose of 1 mg/kg, isolated rat liver cells were prepared as described by Berg and Blomhoff (1983). Parenchymal cells (PC) and non-parenchymal cells (NPC) were isolated from the total liver cell suspension by differential centrifugation. From the non-parenchymal cell suspension, endothelial and Kupffer cells were purified by centrifugal elutriation method (Praaning-Van Daren and Knook, 1982). Four successive fractions were collected at a constant rotor speed of 2500 rev./min: lymphocyte, endothelial cell, intermediate cell and Kupffer cell fractions at flow rates of 14.0, 21.0, 26.0, 40.0 ml/min, respectively. The amount of [^{111}In]Suc-BSA in the liver was estimated from the total number of each cell type contained in 1 g liver (1.25×10^8, 4.2×10^7 and 1.2×10^7 cells/g liver for parenchymal cells, endothelial cells, and Kupffer cells, respectively) (Knook and Brouwer, 1980; Blomhoff et al., 1985b). The purity of separated endothelial cells and Kupffer cells were checked morphologically and enzymatically by measuring peroxidase activity using 3,3-diaminobenzidine as a substrate.

2.7. Liver perfusion experiment

The method for in situ liver perfusion was reported previously (Nishida et al., 1990). The liver was perfused in single-pass mode at a flow rate of 13 ml/min with Krebs–Ringer bicarbonate buffer with 10 mM glucose (oxygenated with 95% O_2/5% CO_2 to pH 7.4 at 37°C). To prevent interactions between macromolecules and blood components, perfusate without red blood cells or albumin was employed. After a stabilization period of 30 min, ^{111}In-labeled anionized proteins dissolved in the perfusate (0.01–10 μg/ml) was infused for 60 min. In the inhibition studies, NH_4Cl (20 mM), DNP (100 μM), cytochalasin B (2 μM), Mal-BSA (1.0 μg/ml), or DS (1.0 μg/ml)

Table 3
In vivo cellular uptake of [^{111}In]Suc-BSA in the liver at 1 h after intravenous injection

	% of dose/10^8cells	Uptake (μg/10^8 cells)	Uptake amount (μg/g liver)
Hepatocyte	0.0954 ± 0.0378	0.238 ± 0.0944	0.298 ± 0.118
Endothelial cells	8.97 ± 1.32	22.4 ± 3.31	9.42 ± 1.39
Kupffer cells	2.68 ± 0.957	6.71 ± 2.39	0.805 ± 0.287

Values are means ± S.D. of at least three experiments.

was also added to the perfusate. The venous outflow and bile were collected into weighed tubes at appropriate intervals. On completion of infusion, perfusate without ^{111}In-labeled anionized proteins was infused for 5 min to wash out the remaining ^{111}In-labeled anionized proteins from the intravascular and Disse spaces. Then, the remaining radioactivity in the liver was measured. The viability of the liver was checked with respect to both the bile flow and the glutamic oxaloacetic transaminase activity in the outflow. In all experiments, perfused livers remained viable during the course of the study.

From the venous outflow curves, the extraction ratio (E_{ss}) and the hepatic clearance (CL_h) at steady state for ^{111}In-labeled anionized proteins are calculated as follows:

$$E_{ss} = \frac{C_{in} - C_{out}}{C_{in}} \quad (1)$$

$$CL_h = E_{ss} \cdot Q \quad (2)$$

where $C_{in,\,ss}$ and $C_{out,\,ss}$ are the respective ^{111}In-labeled anionized protein concentrations in the perfusate before and after passing through the liver under steady-state conditions and Q is the perfusion rate.

2.8. Determination of surface-bound and internalized [^{111}In]Suc-BSA (acid-wash method)

Detachment of [^{111}In]Suc-BSA from the surface of the liver tissue was carried out by the acid wash method. [^{111}In]Suc-BSA (0.1 μg/ml) was infused for 2–20 min and then the ice-cold buffer (4°C) was infused for another 5 min to wash out the remaining [^{111}In]Suc-BSA from the intravascular and Disse spaces. Perfusion medium was then switched to acid buffer (pH 3.0), and effluent was immediately collected every 30 s for 10 min. The acid buffer contained (in mM) 60 CH_3COOH, 60 NaCl, 4.7 KCl, 3.4 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 34.5 $NaHCO_3$, and 7.4 glucose. The amount of surface-bound [^{111}In]Suc-BSA was estimated from the radioactivity recovered in the outflow. After acid wash treatment, the remaining radioactivity in the liver (internalized ligand) was determined.

2.9. Estimation of the internalization rate constant

The internalization rate constant (k_{int}) can be calculated from the slope of the line described by the following equation:

$$[LR_i]_t = k_{int} \cdot \int_0^t [LR_s]\, dt \quad (3)$$

where $[LR_s]$ and $[LR_i]$ are the amount of the ligand-receptor complex at the cell surface and in the intracellular space, respectively, assuming first-order kinetics for internalization. $\int_0^t [LR_s]\, dt$ may be calculated by the trapezoidal method (Yamaoka et al., 1978).

2.10. Pharmacokinetic analysis

Fig. 1 shows a physiological one-organ model employed for the present pharmacokinetic analysis (Nishida et al., 1992). The sinusoidal compartment including the Disse space is assumed to be under conditions of thorough stirring with a concentration equal to that in the outflow (C_s). The terms C_s and C_b shown in Fig. 1 correspond to C_{out} and C_{in}, respectively. The binding compartment is characterized by a maximum binding amount X_∞ and binding constant K and instanta-

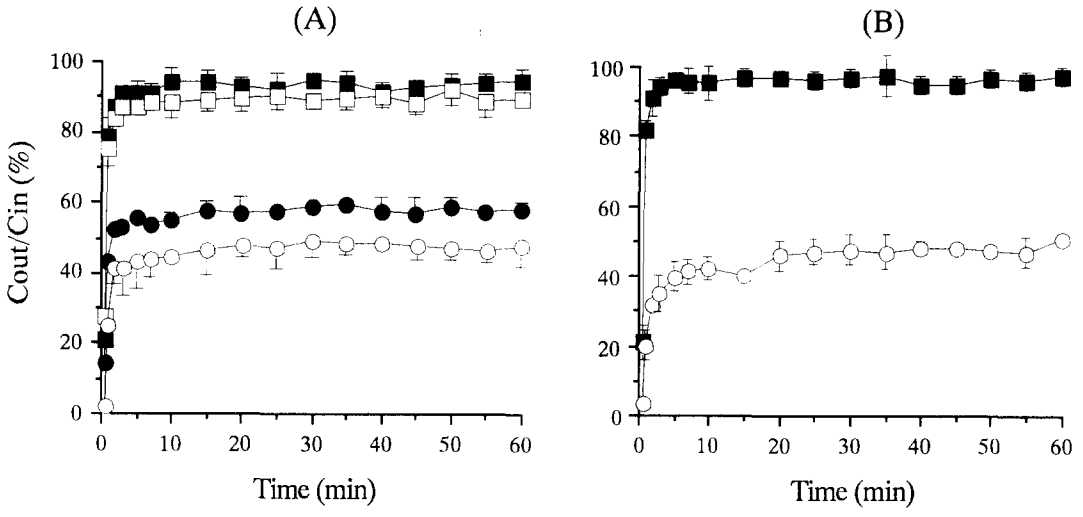

Fig. 3. Hepatic recovery ratio (C_{out}/C_{in})–time profiles of [^{111}In]succinylated proteins (■, Suc-SOD; □, Suc-LZM; ●, Suc-CAT; ○, Suc-BSA) (A) and maleylated proteins (■, Mal-SOD; ○, Mal-BSA) (B) in the isolated rat liver perfusion system at an inflow concentration of 0.1 μg/ml. Values are means of three experiments, with the vertical bars indicating the S.D.

neous equilibration is assumed to occur between the sinusoidal and binding compartments. V_s represents the sum of the volumes of the sinusoid and Disse spaces and as its value we took the figure of 0.180 ml/g liver which was determined in the indicator dilution experiment (Nishida et al., 1990). Assuming first-order kinetics for the internalization process, the internalization rate (dX/dt) is expressed as a product of binding amount X and its rate constant k_{int}. Then, in the sinusoidal and binding compartments, mass–balance equations are defined as follows:

$$V_s\left(\frac{dC_s}{dt}\right)+\left(\frac{dX}{dt}\right)=Q\cdot C_b-Q\cdot C_s-k_{int}\cdot X \quad (4)$$

Binding of BSA derivatives to the cell surface is consistent with the Langmuir equation and the following expression folds.

$$X=\frac{X_\infty\cdot K\cdot C_s}{1+K\cdot C_s} \quad (5)$$

Differentiated with respect to t, Eq. (5) is rearranged to

$$\frac{dX}{dt}=\frac{X_\infty\cdot K}{(1+K\cdot C_s)^2}\cdot\frac{dC_s}{dt} \quad (6)$$

To substitute Eq. (4) with Eq. (6) gives the following equation:

$$\left(V_s+\frac{X_\infty\cdot K}{(1+K\cdot C_s)^2}\right)\cdot\left(\frac{dC_s}{dt}\right)$$
$$=Q\cdot C_b-Q\cdot C_s-\left(\frac{k_{int}\cdot X_\infty\cdot K\cdot C_s}{1+K\cdot C_s}\right) \quad (7)$$

The differential equations derived from Eq. (7) for various inflow concentrations are numerically solved using the Runge-Kutta-Gill method. The initial condition is $C_s=0$ when $t=0$. The fitting was performed by use of the MULTI (RUNGE) program (Yamaoka et al., 1983), which was developed on the M-382 main-frame computer of the Kyoto University Data Processing Center. This program is written in Fortran 77.

3. Results

3.1. Biodistribution of [^{111}In]Suc-BSA after intravenous injection in rats

The biodistribution of [^{111}In]Suc-BSA was determined in rats after intravenous injection at doses of 1 and 50 mg/kg. Fig. 2 shows the plasma

Table 4
Hepatic uptake data of ^{111}In-labeled anionized proteins at an inflow concentration of 0.1 μg/ml in the constant infusion experiment

Compound	Outflow		Amount recovery in the liver (μg)
	E_{ss} (%)	CL_h (μl/min)	
Suc-LZM	10.7 ± 3.4	1380 ± 450	2.2 ± 0.53
Suc-SOD	6.5 ± 3.1	840 ± 400	0.50 ± 0.012
Suc-BSA	52.4 ± 3.9	6810 ± 510	37.4 ± 1.7
Suc-CAT	42.1 ± 2.7	5480 + 350	29.2 ± 3.2
Mal-SOD	2.2 ± 0.96	515 ± 310	2.16 ± 0.96
Mal-BSA	36.9 ± 1.9	6830 ± 370	36.9 ± 1.9

Values are means ± S.D. of at least three experiments.

concentration–time curve of [^{111}In]Suc-BSA. A rapid and biphasic elimination from plasma was observed at a dose of 1 mg/kg and the higher dose gave prolonged plasma retention. The liver was the major contributor for the elimination and the amount of [^{111}In]Suc-BSA recovered in the liver at 6 h was 52% and 28% of dose at doses of 1 and 50 mg/kg, respectively (data not shown). Significant amounts of radioactivity were also detected in the spleen and kidney. Table 2 summarizes the pharmacokinetic parameters for [^{111}In]Suc-BSA. The CL_{total} and CL_{liver} were significantly decreased at high doses, suggesting a saturable uptake process in the liver.

3.2. Cellular distribution of [^{111}In]Suc-BSA after intravenous injection

The cellular distribution of [^{111}In]Suc-BSA in the liver between parenchymal cells, endothelial cells, and Kupffer cells 1 h after intravenous injection at a dose of 1 mg/kg is summarized in Table 3. A significant uptake of [^{111}In]Suc-BSA by liver non-parenchymal cells especially by endothelial cells was observed. The endocytic capacity of endothelial cells for Suc-BSA was about 3.3 times greater than that of Kupffer cells on a cell number basis. Since the number of endothelial cells in the liver is about three times larger than that of Kupffer cells, the contribution of endothelial cells

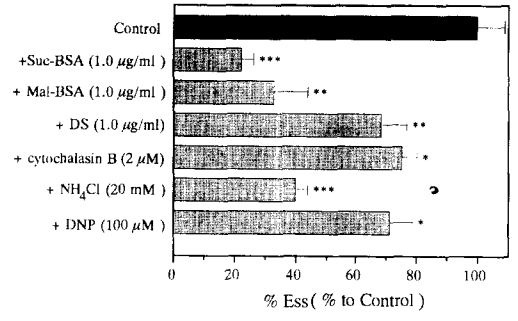

Fig. 4. Competition of hepatic uptake of [^{111}In]Suc-BSA (0.1 μg/ml) under various experimental conditions in the isolated rat liver perfusion system. Values are means of three experiments, with the vertical bars indicating the S.D. * $p < 0.02$, ** $p < 0.01$, *** $p < 0.001$.

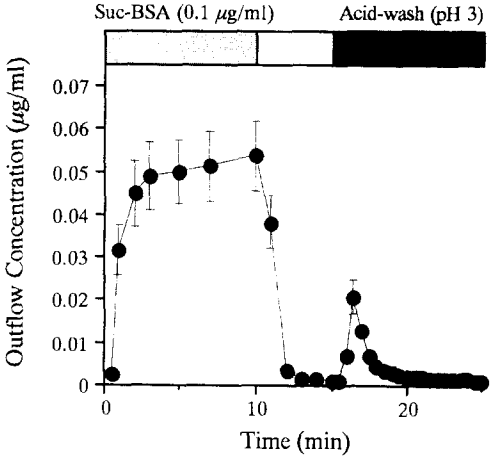

Fig. 5. Representative outflow concentration–time profiles of [^{111}In]Suc-BSA during constant infusion at an inflow concentration of 0.1 μg/ml for 10 min and washout profile into effluent by ice-cold Krebs–Ringer bicarbonate buffer for 5 min followed by acid buffer for 10 min in the isolated rat liver perfusion system. Values are means of three experiments, with the vertical bars indicating the S.D.

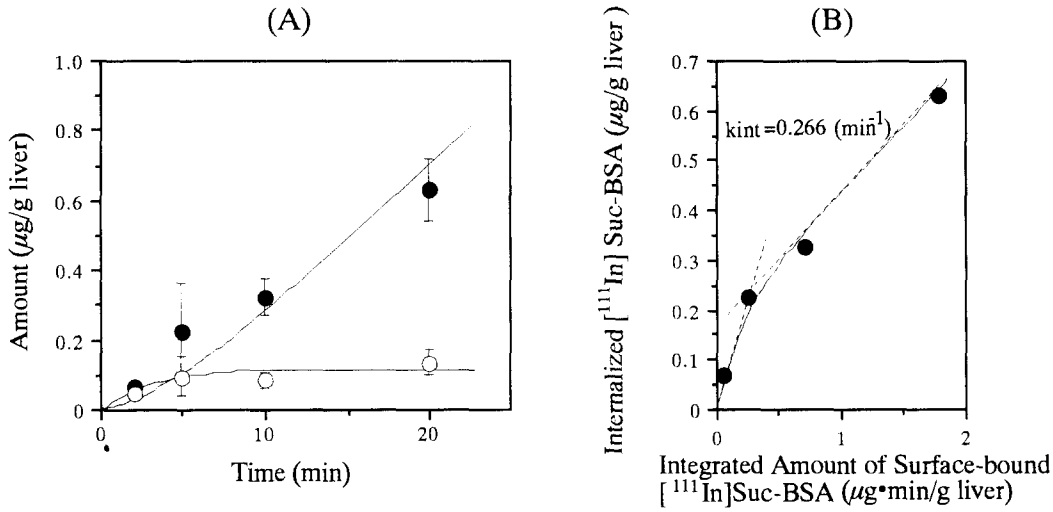

Fig. 6. Time course of the amount of surface-bound (○) and internalized (●) [^{111}In]Suc-BSA in the isolated rat liver perfusion system (A) and plot of [^{111}In]Suc-BSA internalized in the liver versus integrated amount of surface-bound [^{111}In]Suc-BSA (B). Values are means of three experiments, with the vertical bars indicating the S.D.

to the total in vivo hepatic uptake of [^{111}In]Suc-BSA was estimated to be about 12 times higher than that of Kupffer cells.

3.3. Hepatic uptake during constant infusion of ^{111}In-labeled anionized proteins in perfused rat liver

Fig. 3 illustrates the hepatic recovery ratio

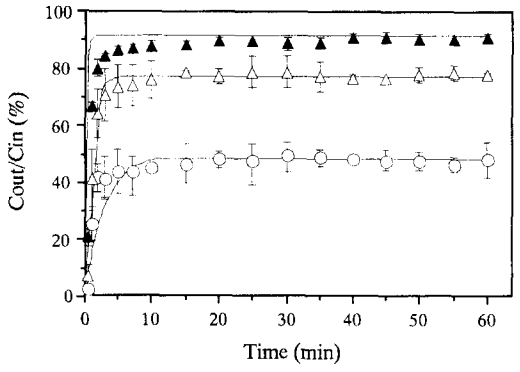

Fig. 7. Hepatic recovery ratio (C_{out}/C_{in})–time profiles of [^{111}In]Suc-BSA in the isolated rat liver perfusion system at an inflow concentration of 0.1 μg/ml (○), 1.0 μg/ml (△), and 10 μg/ml (▲). Values are means of three experiments, with the vertical bars indicating the S.D. Solid lines are simulation curves for the hepatic recovery ratio–time profiles of [^{111}In]Suc-BSA which have been reconstructed employing the estimated parameters shown in Table 5.

(C_{out}/C_{in})–time profiles of [^{111}In]Suc-LZM, Suc-SOD, Suc-BSA, Suc-CAT, Mal-SOD, and Mal-BSA at an inflow concentration of 0.1 μg/ml in the rat liver constant infusion experiment. In all cases, the recovery ratio reached a plateau level within 5 min. [^{111}In]Suc-BSA, [^{111}In]Suc-CAT, and [^{111}In]Mal-BSA were continuously extracted by the liver to a great extent at the steady state while the extraction of [^{111}In]Suc-LZM, [^{111}In]Suc-SOD, and [^{111}In]Mal-SOD was small. The extraction of native proteins were also small; E_{ss} were ≈2, 3 and 20% for SOD, BSA and CAT, respectively. The E_{ss}, CL_h and the recoveries in the liver and bile are summarized in Table 4. E_{ss} of [^{111}In]Suc-BSA was significantly decreased to 23, 33, and 68% of the control values in the presence of 1.0 μg/ml Suc-BSA, Mal-BSA, and DS (8 kDa), respectively (Fig. 4). Treatment with NH_4Cl, dinitrophenol (DNP), and cytochalasin B also decreased the E_{ss} value to 41, 71, and 75% of the control values, respectively.

3.4. Separation of surface-bound and internalized [^{111}In]Suc-BSA by acid-wash method

Fig. 5 shows a representative outflow concentration–time profiles of [^{111}In]Suc-BSA during constant infusion and acid wash experiment in the

Table 5
Pharmacokinetic parameters for hepatic uptake of [^{111}In]Suc-BSA, [^{111}In]Lac-BSA, and [^{111}In]Cat-BSA

Compound	Methods	X_r (μg)	K (ml/μg)	k_{int} (min^{-1})
Suc-BSA	Model analysis[a]	20.3	4.083	0.202
	Experiment (acid-wash)	—	—	0.266
Lac-BSA	Model analysis[b]	38.8	0.868	0.344
	Experiment (EDTA-wash)[b]	40.1	0.568	0.463
Cat-BSA	Model analysis[b]	4186.3	0.176	0.05
	Experiment[b]	49 212.4	0.066	0.015

[a] Parameters were obtained from simultaneous multiple curve fitting to the experimental results at various inflow concentrations by use of the MULTI(RUNGE) program.
[b] X_r, K, and k_{int} were referred from a previous study (Nishida et al., 1992).

isolated rat liver perfusion system. [^{111}In]Suc-BSA was infused for 10 min at an inflow concentration of 0.1 μg/ml, and [^{111}In]Suc-BSA remaining in the extracellular space was removed by washing with Krebs–Ringer bicarbonate buffer (pH 7.4). Then the surface-bound [^{111}In]Suc-BSA was released by washing with acid buffer (pH 3.0).

3.5. Estimation of internalization rate constant

Fig. 6A shows the time courses of the surface-bound and internalized amounts of [^{111}In]Suc-BSA. The surface-bound amount was almost constant after 5 min, while a linear increase was observed in the internalized amount over the 20-min period of measurement. Fig. 6 shows a plot of $[LR_i]$ versus $\int [LR_s]\, dt$ values according to Eq. (3). It showed biphasic increment, and the k_{int} for [^{111}In]Suc-BSA was calculated to be 0.27 min^{-1}.

3.6. Simultaneous multiple curve-fitting to outflow pattern

Pharmacokinetic parameters representing the binding to the cell surface and internalization were calculated according to the simultaneous multiple curve fitting to the experimental results at various inflow concentrations of [^{111}In]Suc-BSA (Fig. 7). Table 5 summarizes the obtained pharmacokinetic parameters for [^{111}In]Suc-BSA together with those for [^{111}In]Lac-BSA and [^{111}In]Cat-BSA (Nishida et al., 1992). Fig. 7 also shows simulation curves for the hepatic recovery ratio–time profiles of [^{111}In]Suc-BSA which have been reconstructed employing the estimated parameters. In general, good agreement was observed between fitted curves and experimentally observed data at all inflow concentrations. As for [^{111}In]Suc-BSA, its surface-bound and internalized amounts at respective times of 5, 20, and 60 min after the start of perfusion of [^{111}In]Suc-BSA (0.1 μg/ml) obtained with the acid wash experiment, correlated well with those calculated from the present kinetic parameters (Table 5). This finding might support the validity of the present fitting procedure. At the initial stage of hepatic uptake within 5 min after the start of infusion, however, some diversities are observed between them.

4. Discussion

Our recent work has demonstrated that succinylated proteins are preferentially taken up by liver non-parenchymal cells via a scavenger receptor-mediated mechanism after intravenous injection in mice (Takakura et al., 1994). The purpose of the present study was to characterize the hepatic scavenger receptor-mediated uptake of anionized proteins in more detail. The organ perfusion system using isolated rat liver is a powerful tool to study the pharmacokinetic characteristics of

macromolecules at the organ level (Nishida et al., 1992).

Prior to the organ perfusion experiments, we studied in vivo pharmacokinetics of [^{111}In]Suc-BSA after intravenous injection in rats. The results have shown that the rat liver plays an important role in the elimination of [^{111}In]Suc-BSA in the circulation and takes up [^{111}In]Suc-BSA in a dose dependent manner (Fig. 2, Table 2), as shown in mice (Takakura et al., 1994). In addition, hepatic cellular localization experiments using centrifugal elutriation technique have demonstrated that sinusoidal endothelial cells contribute to the greatest extent among the hepatic cell populations. This finding corresponds well to the immunohistochemical observation by Jansen et al. (1993b) for succinylated human serum albumin (Suc-HSA) in rats. Our quantitative evaluation has revealed that the contribution of the endothelial cells to the hepatic uptake of Suc-BSA in vivo was about 12 times higher than that of the Kupffer cells (Table 3).

At present, macrophage scavenger receptors are the most well-characterized scavenger receptors, trimeric membrane glycoproteins (Krieger and Herz, 1994; Pearson, 1996). The macrophage scavenger receptors are expressed only on Kupffer cells in the liver but not on sinusoidal endothelial cells. On the other hand, the liver sinusoidal endothelial cells have scavenger receptors for polyanions such as acetyl-LDL (Blomhoff et al., 1985a; Pitas et al., 1985) formaldehyde-treated albumin (Jansen et al., 1991b) in vivo, but both ligands are reportedly recognized by different receptors (Horiuchi et al., 1985). Recent studies have suggested the existence of various scavenger receptors on Kupffer and liver endothelial cells (Ottnad et al., 1990; Van Berkel et al., 1991; Bickel and Freeman, 1992). It is well known that the liver endothelial cells have the receptors for hyaluronan, a kind of polyanion (Smedsrod et al., 1990). However, the relationship between these receptors with scavenger functions for polyanionic compounds remains to be elucidated. Little is known about the uptake characteristics by the scavenger receptors on the liver endothelial cells.

In the rat liver perfusion experiment, significant hepatic uptake of Suc-BSA, Suc-CAT, and Mal-BSA was observed whereas Suc-LZM, Suc-SOD, and Mal-SOD were not effectively taken up by the liver (Fig. 3). There was no significant difference in the uptakes of succinylated and maleylated proteins. Similar molecular weight-dependent uptake was shown in in vivo studies in mice (Takakura et al., 1994) and in vitro studies using cultured brain microvessel endothelial cells (Tokuda et al., 1993). These results suggested the importance of molecular weight or total number of anionic charges per one protein molecule for the recognition by the scavenger receptor on the liver endothelial cells.

The hepatic uptake of Suc-BSA in the constant infusion experiment was saturable process (Fig. 3) and inhibited by coadministration of NH_4Cl, DNP, or cytochalasin B (Fig. 4), suggesting a receptor-mediated endocytosis of the ligand. Simultaneous injection with typical and reliable ligands for scavenger receptors, Mal-BSA and DS also significantly reduced the hepatic uptake of [^{111}In]Suc-BSA (Fig. 4). These results suggested that [^{111}In]Suc-BSA underwent binding to the scavenger receptors on the cells and subsequent internalization.

In order to analyze the hepatic uptake processes via the scavenger receptors, we carried out two approaches, acid-wash method and model analysis using a physiological one-organ pharmacokinetic model. With acid-wash treatment, the surface-bound amount was almost constant after 5 min, while a linear increase was observed in the internalized amount over the 20-min period of measurement (Fig. 6). According to Eq. (3), the k_{int} for [^{111}In]Suc-BSA was calculated to be 0.27 min^{-1}, suggesting that [^{111}In]Suc-BSA was internalized very effectively by the scavenger receptors on the liver endothelial cells. The internalization of [^{111}In]Suc-BSA by the scavenger receptor showed biphasic increment. The reason is not clear at present and further assessment will be required for this phenomenon.

In another method, the outflow concentration–time profiles at three different inflow concentrations were analyzed based on a physiological one-organ model in which binding and internalization were assessed separately (Fig. 1). As shown in Fig. 7, the computer fitting yielded good

results at any inflow concentrations, suggesting the validity of this model. However, in the early phase after the start of infusion, the model did not fit the data well. In this analysis, it is assumed that the sinusoidal space is under conditions of thorough stirring and that the binding to the cell surface occurs instantaneously. In a strict sense, such a discrepancy might be caused by the inapplicability of these conditions. Hepatic extraction of [^{111}In]Suc-BSA in the early phase may be so extensive that the sinusoidal concentrations declined with distance along blood flow.

The calculated pharmacokinetic parameters of [^{111}In]Suc-BSA, [^{111}In]Lac-BSA, and [^{111}In]Cat-BSA were greatly different between the compounds. For [^{111}In]Cat-BSA, indeed there are differences between the parameters calculated by the model analysis and experiment, and this inconsistency may imply that the physiological model used in this study (Fig. 1) is not appropriate for [^{111}In]Cat-BSA. However, we tried to characterize the differences in hepatic uptake processes of these compounds on a basis of the same physiological one-organ pharmacokinetic model. In comparison with [^{111}In]Lac-BSA and [^{111}In]Cat-BSA, the binding site affinity (K) of [^{111}In]Suc-BSA was much higher, and internalization rate constant (k_{int}) of [^{111}In]Suc-BSA was as high as [^{111}In]Lac-BSA, whereas the binding capacity (X_∞) was approx. 1/200 of that of [^{111}In]Cat-BSA (Table 5). These differences on pharmacokinetic parameters reflect the differences of hepatic uptake mechanism of these BSA derivatives; asialoglycoprotein receptor-mediated endocytosis, adsorptive-mediated endocytosis and scavenger receptor-mediated endocytosis for [^{111}In]Lac-BSA, [^{111}In]Cat-BSA and [^{111}In]Suc-BSA, respectively. Thus the analysis has clarified that the pharmacokinetics in the hepatic uptake behaviors of these BSA derivatives differed greatly at the suborgan level. A precise understanding of these differences would lead to more rational design of drug targeting using these macromolecules as carriers.

Jansen et al. (1991a, 1993a) has reported that Suc-HSA exhibits a potent anti-HIV-1 activity in vitro. However, rapid clearance by the liver endothelial cells after systemic administration may limit its use as anti-HIV-1 agent. The present study has demonstrated that large succinylated and maleylated proteins should be useful as a carrier for the intracellular delivery of drugs specifically into the liver endothelial cells. Direct anionization may be also promising for the intracellular targeting of protein drugs.

References

Arano, Y., Mukai, T., Uezono, T., Wakisaka, K., Motonari, H., Akizawa, H., Taoka, Y. and Yokoyama, A., A biological method to evaluate bifunctional chelating agents to label antibodies with metallic radionuclides. *J. Nucl. Med.*, 35 (1994) 890–898.

Berg, T. and Blomhoff, R., Preparation of isolated rat-liver cells. *Iodinated Density Gradient Media.*, (1983) 173–174.

Bickel, P.E. and Freeman, M.W., Rabbit aortic smooth muscle cells express inducible macrophage scavenger receptor messenger RNA that is absent from endothelial cells. *J. Clin. Invest.*, 90 (1992) 1450–1457.

Blomhoff, R., Dreron, C., Eskid, W., Helgerd Norum, K.R. and Berg, T., Clearance of acetyl low density lipoprotein by rat liver endothelial cells. *J. Biol. Chem.*, 259 (1985a) 8898–8903.

Blomhoff, R., Blomhoff, H.K., Tolleshaug, H., Christensen, T.B. and Berg, T., Uptake and degradation of bovine testes β-galactosidase by parenchymal and nonparenchymal rat liver cells. *Int. J. Biochem.*, 17 (1985b) 1321–1328.

Duncan, J.R. and M.J. Welch. Intracellular metabolism of indium-111-DTPA-labeled receptor targeted proteins. *J. Nucl. Med.*, 34 (1993) 1728–1738.

Fujita, T., Nishikawa, M., Tamaki, C., Takakura, Y., Hashida, M. and Sezaki, H., Targeted delivery of human recombinant superoxide dismutase by chemical modification with mono- and polysaccharide derivatives. *J. Pharmacol. Exp. Ther.*, 263 (1992a) 971–978.

Fujita, T., Furitsu, H., Nishikawa, M., Takakura, Y., Sezaki, H. and Hashida, M., Therapeutic effects of superoxide dismutase derivatives modified with mono- or polysaccharides on hepatic injury induced by ischemia/reperfusion. *Biochem. Biophys. Res. Commun.*, 189 (1992b) 191–196.

Habeeb, A.F.S.A., Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. *Anal. Biochem.*, 14 (1966) 328–336.

Hirabayashi, H., Nishikawa, M., Takakura, Y. and Hashida, M., Development and pharmacokinetics of galactosylated poly-L-glutamic acid as a biodegradable carrier for liver-specific drug delivery. *Pharm. Res.*, 13 (1996) 880–884.

Hnatowich, D., Layne, W.W. and Childs, R.L., The preparation and labeling of DTPA-coupled albumin. *Int. J. Appl. Radiat. Isot.*, 12 (1982) 327–332.

Horiuchi, S., Takata, K., Maeda, H. and Morino, Y., Scavenger function of sinusoidal liver cells. Acetylated low-den-

sity lipoprotein is endocytosed via a route distinct from formaldehyde-treated serum albumin. *J. Biol. Chem.*, 259 (1985) 53–56.

Hugo, A., Catalase in vitro. *Methods Enzymol.*, 105 (1984) 121–126.

Jansen, R. W., Molema, G., Pauwels, R., Schols, D., De Clercq, E. and Meijer, D.K.F., Potent in vitro anti-human immunodeficiency virus-1 activity of modified human serum albumins. *Mol. Pharmacol.*, 39 (1991a) 818–823.

Jansen, R.W., Molema, G., Harms, G., Kruit, J.K., Hardonk, M.J. and Meijer, D.K., Formaldehyde treated albumin contains monomeric and polymeric forms that are differently cleared by endothelial and Kupffer cells of the liver: evidence for scavenger receptor heterogeneity. *Biochem. Biophys. Res. Commun.*, 180 (1991b) 23–32.

Jansen, R.W., Pauwels, R., Schols, D., De Clercq, E. and Meijer, D. K.F., Novel, negatively charged, human serum albumins display potent and selective in vitro anti-human immunodeficiency virus type 1 activity. *Mol. Pharmacol.*, 44 (1993a) 1003–1007.

Jansen, R.W., Peter, O., Geert, H. and Meijer, D.K.F., Pharmacokinetic analysis and cellular distribution of the anti-HIV compound succinylated human serum albumin (Suc-HSA) in vivo and in the isolated perfused rat liver. *Pharm. Res.*, 10 (1993b) 1611–1614.

Knook, D.L. and Brouwer, A., *The Reticuloendothelial System and the Pathogenesis of Liver Disease*, Elsevier/North-Holland, Amsterdam, 1980, pp. 17–25.

Krieger, M. and Herz, J., Structures and functions of multiligand lipoprotein receptors: macrophage scavenger receptors and LDL receptor-related protein (LRP). *Annu. Rev. Biochem.*, 63 (1994) 601–637.

Nishida, K., Tonegawa, C., Nakane, S., Takakura, Y., Hashida, M. and Sezaki, H., Effect of electric charge on the hepatic uptake of macromolecules in the rat liver. *Int. J. Pharm.*, 65 (1990) 7–17.

Nishida, K., Mihara, K., Takino, T., Nakane, S., Takakura, Y., Hashida, M. and Sezaki, H., Hepatic disposition characteristics of electrically charged macromolecules in rat in vivo and in the perfused liver. *Pharm. Res.*, 8 (1991) 437–444.

Nishida, K., Takino, T., Eguchi, Y., Yamashita, F., Hashida, M. and Sezaki, H., Pharmacokinetic analysis of uptake process of lactosaminated albumin in the rat liver constant infusion experiments. *Int. J. Pharm.*, 80 (1992) 101–108.

Nishikawa, M., Kamijo, A., Fujita, T., Takakura, Y., Sezaki, H. and Hashida, M., Synthesis and pharmacokinetics of a new liver-specific carrier, glycosylated carboxymethyl-dextran, and its application to drug targeting. *Pharm. Res.*, 10 (1993) 1253–1264.

Ottnad, E., Via, D.P., Sinn, H., Friedrich, E., Ziegler, R. and Dresel, H.A., Binding characteristics of reduced hepatic receptors for acetylated low-density lipoprotein and maleylated bovine serum albumin. *Biochem. J.*, 265 (1990) 689–698.

Pearson, A.M., Scavenger receptors in innate immunity. *Curr. Opin. Immunol.*, 8 (1996) 20–28.

Pitas, R.E., Boyles, J., Mahley, R.W. and Bissell, D.M., Uptake of chemically modified low density lipoproteins in vivo is mediated by specific endothelial cells. *J. Cell Biol.*, 100 (1985) 103–117.

Praaning-Van Daren, D.P. and Knook, D.L., Quantitative determination of in vivo endocytosis by rat liver Kupffer and endothelial cells facilitated by an improved cell isolation method. *FEBS Lett.*, 141 (1982) 229–232.

Smedsrod, B., Pertoft, H., Gustafson, S. and Laurent, T.C., Scavenger functions of the liver endothelial cells. *Biochem. J.* 266 (1990) 313–327.

Takakura, Y., Takagi, A., Hashida, M. and Sezaki, H., Disposition and tumor localization of mitomycin C-dextran conjugates in mice. *Pharm. Res.*, 4 (1987) 293–300.

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

Takakura, Y., Fujita, T., Furitsu, H., Nishikawa, M., Sezaki, H. and Hashida, M., Pharmacokinetics of succinylated proteins and dextran sulfate in mice: implications for hepatic targeting of protein drugs by direct succinylation via scavenger receptors. *Int. J. Pharm.*, 105 (1994) 19–29.

Tokuda, H., Masuda, S., Takakura, Y., Sezaki, H. and Hashida, M., Specific uptake of succinylated proteins via a scavenger receptor-mediated mechanism in cultured brain microvessel endothelial cells. *Biochem. Biophys. Res. Commun.*, 196 (1993) 18–24.

Van Berkel, T.J.C., De Rijke, Y.B. and Kruijt, J.K., Different fate in vivo of oxidatively modified low density lipoprotein and acetylated low density lipoprotein in rats. Recognition by various scavenger receptors on Kupffer and endothelial liver cells. *J. Biol. Chem.*, 266 (1991) 2282–2289.

Yamaoka, K., Nakagawa, T. and Uno, T., Statistical moments in pharmacokinetics. *J. Pharmacokinet. Biopharm.*, 6 (1978) 547–55800.

Yamaoka, K. and Nakagawa, T., A nonlinear least squares program based on differential equations, MULTI (RUNGE), for microcomputers. *J. Pharmacobio-Dyn.*, 6 (1983) 595–606.