A publication of the American Pharmaceutical Association and the American Chemical Society

JOURNAL OF Pharmaceutical Sciences

June 1999
Volume 88, Number 6

RESEARCH ARTICLES

Disposition of Radioactivity after Injection of Liver-Targeted Proteins Labeled with ^{111}In or ^{125}I. Effect of Labeling on Distribution and Excretion of Radioactivity in Rats

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Received November 16, 1998. Accepted for publication March 26, 1999.

Abstract □ The effect of radiolabeling liver-specific proteins on the in vivo disposition of radioactivity was investigated. The suitability of ^{111}In and ^{125}I as radiolabels for protein disposition studies in vivo was examined. Galactosylated and cationized bovine serum albumin were labeled with either ^{125}I by the chloramine-T method or ^{111}In, using 1-(4-isothiocyanatobenzyl)ethylenediaminetetraacetic acid (SCN-BZ-EDTA) or diethylenetriaminepentaacetic acid (DTPA) as bifunctional chelating agents (BCAs) and administered intravenously to rats. ^{125}I radioactivity disappeared rapidly from the liver with subsequent excretion in the urine and bile, mainly in the TCA soluble fraction. ^{111}In-associated radioactivity, on the other hand, remained in the hepatic tissue in considerably higher amounts during the experiment and was excreted in the bile and urine to a lower extent when compared with ^{125}I. When the effect of BCA on excretion of ^{111}In radioactivity was compared, no significant differences were observed in the urinary clearances. However, biliary excretion was significantly higher for ^{111}In-SCN-BZ-EDTA-bound radioactivity. In conclusion, when compared with ^{125}I, ^{111}In labeling seems to more accurately characterize the in vivo distribution of liver-targeted proteins after their iv administration in rats and allows a more accurate pharmacokinetic evaluation to be performed.

Introduction

Since biologically active proteins are receiving much attention as potential therapeutic agents, there has been a need to modify their structure in order to overcome difficulties associated with their clinical application. Consequently, it is necessary to quantitatively describe the disposition of these proteins in the body using pharmacokinetic evaluation. Labeling a protein in question with a radionuclide is the easiest way to trace it in the organism, and a variety of methods have been employed to label proteins with a radionuclide. Among these, radioiodine isotopes are the most widely used protein radiolabels for imaging studies.[1] Proteins may be easily labeled with radioisotopes of iodine by several methods; however, certain limitations to the use of radioiodine for imaging applications have become apparent, especially their poor target retention of radioactivity.[2,4]

Several alternative methods of radiolabeling proteins and peptides have been examined to overcome these difficulties and to increase and retain enough radioactivity at target sites for a sufficient period of time. After introduction of bifunctional chelating agents (BCAs), ^{111}In has become a convenient radiometal which has been used by many investigators.[4] This radionuclide is commonly attached to a protein via ethylenediaminetetraacetic acid (EDTA) or, alternatively, via diethylenetriaminepentaacetic acid (DTPA). In addition, several other modifications of these BCAs have been investigated to improve labeling efficiency and in vivo stability.[5−8]

When internalized by cells, these proteins undergo intracellular catabolism with subsequent formation of radiolabeled metabolites. In particular, after endocytotic internalization, the ligand is delivered to the endosome and then to the lysosome. In the lysosomal compartment, most

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10.1021/js9804415 CCC: $18.00
Published on Web 05/06/1999

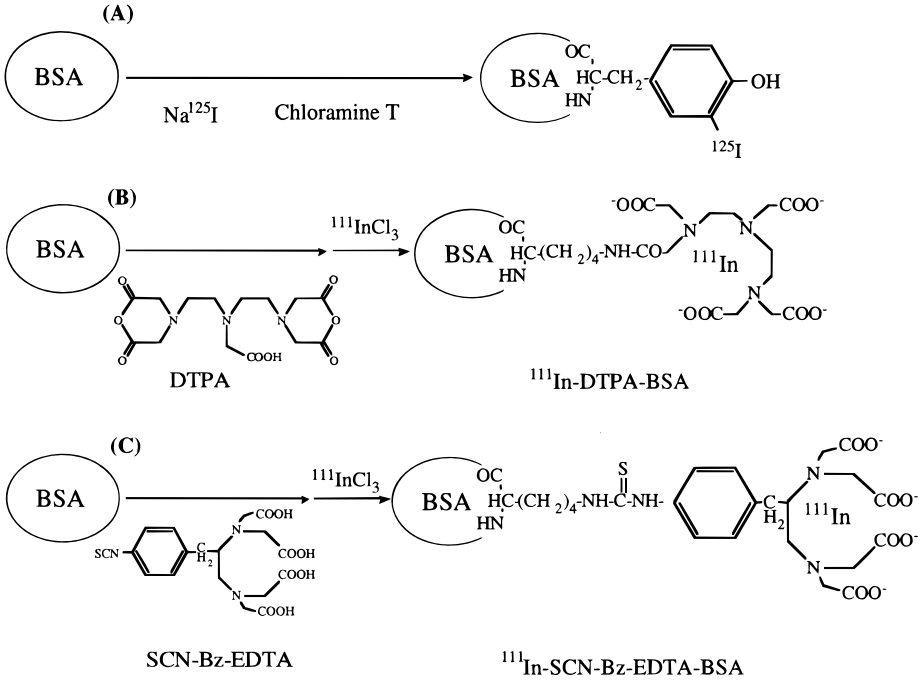

Figure 1—Chemical structures of BSA derivatives radiolabeled with (A) ^{125}I, (B) ^{111}In-DTPA, or (C) ^{111}In-SCN-BZ-EDTA.

of the ligand is degraded by proteases and glycosidases.[9] Obviously, further disposition of radioactivity within the body will be determined by the nature of the radiolabeled degradation product(s) formed, mainly by their physicochemical characteristics such as lipophilicity and molecular weight and/or by their possible affinity for a carrier-mediated transport system. This problem becomes especially marked in cases where proteins are intended to be preferentially taken up by liver cells. In the case of radioiodine-labeled proteins, the final radioactive metabolite is mono- or diiodotyrosine[10] which seems to escape from the liver at a very fast rate with consequent interference with the pharmacokinetic analysis.

The aim of the present study was to investigate and quantitatively compare the difference between ^{125}I and ^{111}In radiolabeling of liver-specific proteins in terms of the whole body disposition of radioactivity. Galactosylated bovine serum albumin (Gal-BSA) and cationized BSA (Cat-BSA) were employed as carrier proteins for hepatic cells. Both have been reported to be taken up preferentially by hepatic parenchymal cells, although the mechanism and rate by which they enter cells differ. Hepatic uptake of Gal-BSA occurs via asialoglycoprotein receptors, whereas Cat-BSA enters hepatocytes by adsorptive endocytosis based on universal electrostatic interaction with the cell surface (for review see refs 11 and 12). In the present study, ^{111}In was attached to the proteins using 1-(4-isothiocyanatobenzyl) EDTA (SCN-BZ-EDTA) or DTPA as chelating agents as described by Meares et al.[13] and Hnatowich et al.,[14] respectively. Radioiodination of both proteins by ^{125}I was achieved by the chloramine-T method.[15] Radiolabeled compounds were administered to rats as a bolus intravenous injection. The radioactivity in the plasma, bile, urine, and several organs was measured, and its movement within the body was described using clearance concepts.

Materials and Methods

Chemicals—BSA (fraction V) was purchased from Sigma Chemicals (St Louis, MO). SCN-BZ-EDTA and DTPA anhydride were obtained from Dojindo Laboratory (Kumamoto, Japan). ^{111}Indium chloride ([^{111}In]$InCl_3$) was supplied from Nihon Medi-Physics Co. (Takarazuka, Japan). Chloramine T was supplied by Nacalai Tesque (Kyoto, Japan). Sodium (^{125}I) iodide ([^{125}I]NaI) was purchased from DuPont/NEN Co. (Boston, MA). All other chemicals were obtained commercially as reagent-grade products.

Synthesis of BSA Derivatives—The method of Lee et al.[16] was used to introduce galactose residues into BSA. Briefly, cyanomethyl 2,3,4,6-tetra-*O*-acetyl-1-thio-D-galactopyranoside was treated with 0.01 M sodium methoxide at room temperature for 24 h, and the syrup of 2-imino-2-methoxyethyl 1-thio-D-galactopyranoside was obtained after evaporation of the solvent. This resultant syrup was quantitatively added to BSA (100 mg) in 10 mL of 50 mM borate buffer (pH 9.0). After 24 h at room temperature, the reaction mixture was concentrated by ultrafiltration and applied to a Sephadex G-25 column equilibrated with 0.1 M acetate buffer (pH 6.0) to remove the unreacted compound. The number of sugars incorporated into the albumin was determined by the phenol/sulfuric acid method calibrated with galactose.[17]

Cat-BSA was synthesized by covalent coupling of hexamethylendiamine to BSA according to the method reported by Pardridge et al.[18] In brief, 5 mL of 10% BSA in distilled water was slowly added to 30 mL of 2 M hexamethylendiamine at pH 6.5. 1-ethyl-(3-dimethylaminopropyl)carboimide hydrochloride (0.5 g) was added to the solution after 30 and 60 min, and the solution was kept at a pH of 6.5 by addition of 1 M HCl. The solution was stirred overnight and then dialyzed thoroughly against distilled water. The protein was purified by chromatofocusing using Polybuffer exchanger 94 resin and Polybuffer 96 elution buffer (Pharmacia, Uppsala, Sweden). The major protein peak eluted in the void volume and was collected and concentrated by ultrafiltration. The cationic nature of Cat-BSA was confirmed by its absorption on a CM-Sephadex C-50 anion exchanger.

^{125}I Labeling—A modified chloramine-T method[15] was used to label both proteins with ^{125}I. Twenty μL 0.4 M phosphate buffer (pH 7.5) and 20 μL 10-fold diluted [^{125}I]NaI were added to 20 μL the sample solution (1 mg/mL) and vortexed. Two times 20 μL chloramine-T diluted in ice-cold distilled water (3.8 mM) was added and vortexed for 30 s. Finally, 50 μL of sodium metabisulfite solution (2.5 mM) was added and vortexed for 30 s. The solution was applied to a PD-10 column (Pharmacia, Uppsala, Sweden) and eluted with phosphate buffer (0.25 M, pH 7.5) containing 0.2% BSA. The eluent was collected into plastic tubes and the radioactivity counted using a survey meter. Appropriate fractions were taken and stored at −80 °C until use. The specific activity of the obtained sample was 74 MBq/mg protein.

^{111}In Labeling of BSA Derivatives—^{111}In labeling of BSA derivatives was performed using DTPA and SCN-BZ-EDTA as the

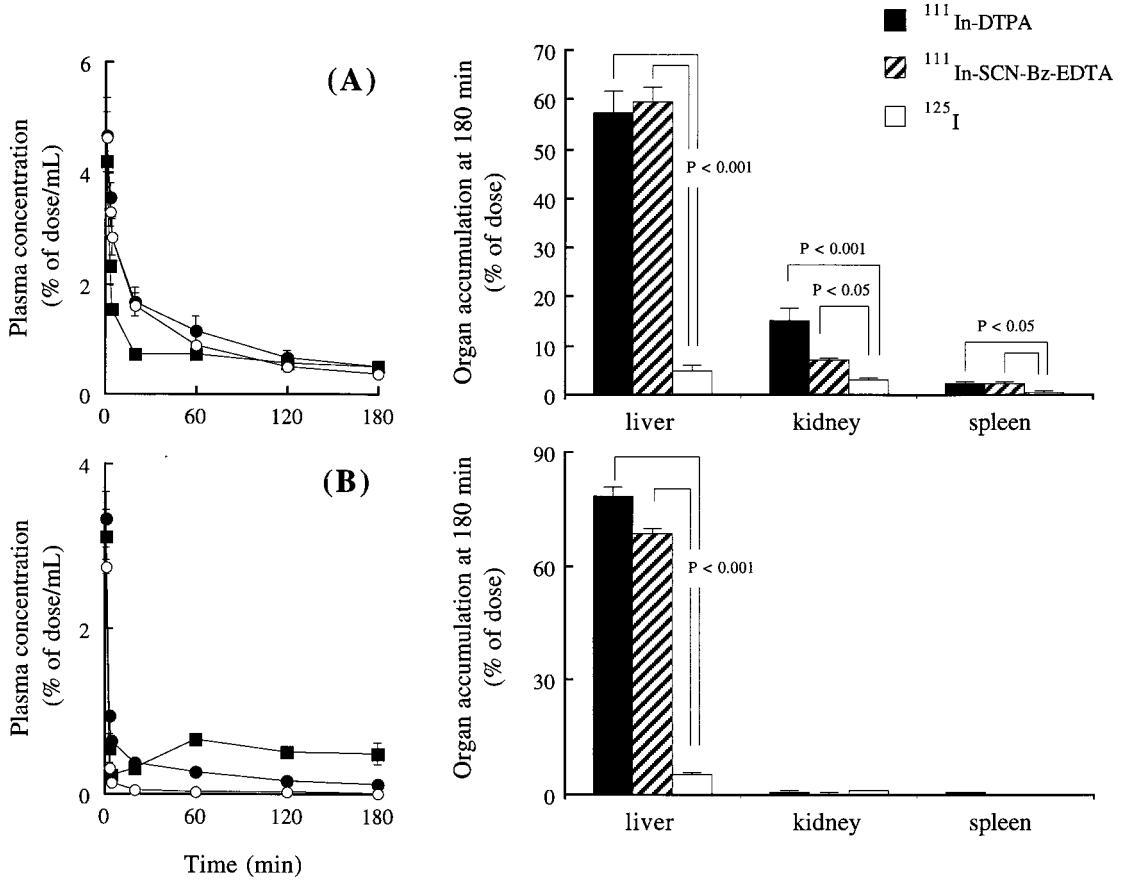

Figure 2—In vivo disposition of radioactivity after iv administration of Cat-BSA (A) and Gal-BSA (B) labeled with ^{111}In-DTPA (●), ^{111}In-SCN-BZ-EDTA (○), or ^{125}I (■). Data are expressed as mean ± SD of at least three experiments. Statistical significance was based on Student *t*-test.

bifunctional chelating agents by the method of Hnatowich et al.[14] and Meares et al.,[13] respectively.

Attachment of DTPA: Gal-BSA or Cat-BSA (2 mg) was dissolved in 1 mL of 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid buffer (0.1 M, pH 7.0), and a 2-fold molar excess of DTPA anhydride in 10 μL of dimethyl sulfoxide was added. After stirring 30 min at room temperature, the mixture was purified by gel-filtration chromatography using a Sephadex G-25 column (1 × 40 cm) and eluted with acetate buffer (0.1 M, pH 6.0) to separate the unreacted DTPA. Fractions containing DTPA−BSA were selected using spectrophotometry and concentrated by ultrafiltration.

Attachment of SCN-BZ-EDTA: Gal-BSA or Cat-BSA (5 mg) was dissolved in 1 mL of borate-buffered saline (50 mM, pH 8.5) and an equimolar amount of SCN-BZ-EDTA in 10 μL of dimethylformamide was added. After 24 h at 37 °C, the mixture was purified by gel-filtration chromatography using a Sephadex G-25 column (1 × 40 cm) and eluted with acetate buffer (0.1 M, pH 3.0) to separate the unreacted SCN-BZ-EDTA. The fractions containing SCN-BZ-EDTA-BSA were treated as described above.

Thirty microliters of $^{111}InCl_3$ solution was added to 30 μL of sodium acetate buffer (1 M, pH 6.0) and 60 μL of either the DTPA- (or SCN-BZ-EDTA)−BSA derivative was added to the mixture. After 30 min at room temperature (45 min at 37 °C, in the case of SCN-BZ-EDTA−BSA), the mixture was purified by gel filtration chromatography using a PD-10 column (Pharmacia, Uppsala, Sweden) and eluted with acetate buffer (0.1 M, pH 6.0). The appropriate fractions were selected based on their radioactivity and concentrated by ultrafiltration. The specific activity of the obtained samples was 37 MBq/mg protein.

In Vivo Animal Experiments—Prior to administration, trace amounts of radiolabeled proteins were diluted with saline, and the protein concentration was adjusted to 0.1 mg/mL by adding nonlabeled protein. Male Wistar rats (200 g) maintained on a standard rat food and water diet were anaesthetized by intraperitoneal administration of pentobarbital sodium at a dose of 50 mg/kg. Their urinary bladder and bile duct were cannulated using polyethylene and polyvinyl tubing to collect bile and urine samples, respectively. Then, 200 μL investigated protein was injected into the femoral vein at a dose of 0.1 mg/kg. Samples of blood, urine, and bile were collected throughout the 180 min of the experimental period. At the end of the experiment, each animal was killed by bleeding and the liver, kidney, and spleen were excised. Blood samples were centrifuged at 2000*g* for 2 min, and 100 μL of plasma was collected for analysis. The ^{111}In and ^{125}I radioactivity in the samples was measured in a well, NaI scintillation counter (ARC-500, Aloka, Tokyo, Japan).

Table 1—Physicochemical Properties of BSA and Its Derivatives Used in This Study

	molecular weight[a]	number of NH_2 groups[b]	number of galactose[c]	pI[d]
BSA	67000	60.0	–	4.2–4.8
Cat-BSA	70000	69.3	–	>9.0
Gal-BSA	69000	8.6	42	4.8–4.9

[a] Determined by size-exclusion chromatography (LC-6A, Shimadzu, Japan) using a Shim-pack Diol-300 column. [b] Determined by the TNBS method. [c] Determined by the phenol–sulfuric acid method. [d] Determined by chromatofocusing.

Organ Distribution of ^{125}I-Labeled BSA Derivatives—Male Wistar rats similar to these defined above were given 0.1 mg/kg ^{125}I-Cat-BSA or ^{125}I-Gal-BSA. Animals were sacrificed at 5, 20, or 60 min by bleeding, the liver, kidney, spleen, and thyroid were excised, and the radioactivity was measured as described above. Data from the interval of 180 min were adopted from the experiments described in the In Vivo Animal Experiments section.

Assessment of Degradation Products of ^{125}I-Labeled Proteins in Plasma, Bile, and Urine—To investigate the degradation products of ^{125}I-labeled proteins, the samples of plasma, bile, and urine were weighed, and an equivalent volume of 45% trichloracetic acid (TCA) was added. Samples were vortexed and centrifuged at 1500*g* for 10 min. Supernatant (100 μL) was separated into different tubes, and the radioactivity of the TCA-precipitable (protein) and TCA-soluble (degradation product) fractions was measured separately as described above.

Table 2—Apparent Volumes of Distribution, Distribution Half-Lives, AUCs, and Clearances of Cat-BSA and Gal-BSA Based on Radioactivity Concentrations/Amounts in Body Compartments[a,b]

compound	method of labeling	V_d (mL)	$t_{1/2}$ of distribution phase (min)	AUC (% of dose·min·mL^{-1})	CL_{total} (mL·h^{-1})	CL_{liver} (mL·h^{-1})	CL_{kidney} (mL·h^{-1})	CL_{spleen} (mL·h^{-1})	$CL_{thyroid}$ (mL·h^{-1})
Cat-BSA	^{111}In-DTPA	17.3	2.89*	244***,†	24.6***	17.8***	4.73††	0.71	—
	^{111}In-SCN-BZ-EDTA	17.7	2.87*	194***	31.0***	21.7***	2.51**	0.77	—
	^{125}I	16.1	1.76	42.5	141	7.07	4.42	0.83	1.55
Gal-BSA	^{111}In-DTPA	10.5	0. 831	12.3	487	382***	3.41**,††	—	—
	^{111}In-SCN-BZ-EDTA	10.9	0.613	15.0	401	324***	0.98***	—	—
	^{125}I	12.2	0.790	14.7	407	31.6	5.62	—	11.2

[a] Results of ^{125}I are based on the TCA-precipitable fractions of radioactivity in the plasma. [b] Statistical significance was based on Student *t*-test. * Compared with ^{125}I, † compared with ^{111}In-SCN-BZ-EDTA, *(†) $p < 0.05$, **(††) $p < 0.01$, *** $p < 0.001$.

Data Analysis—In this study, the radioactivity of particular samples and organs was employed to express the apparent organ uptake clearance of investigated proteins. The radioactivity of all samples and that of plasma was normalized to the % dose or % dose/mL, respectively. Plasma concentrations ($C_p(t)$) were analyzed by mono- or biexponential functions using the nonlinear least-squares program MULTI.[19] The one- or two-compartment model was chosen according to Akaike Information Criterion.

Total body clearance of radioactivity (CL_{total}) was calculated by:

$$CL_{total} = \frac{D}{AUC} \quad (1)$$

where D is the dose of radioactivity administered, and AUC is the area under the plasma concentration−time curve extrapolated to infinity.

Assuming zero or negligible leakage of radioactivity from organs, the apparent organ uptake clearance (CL_{organ}) may be expressed as:

$$CL_{organ} = \frac{X_{t_i}}{AUC_{t_0-t_i}} \quad (2)$$

where X_{t_i} is the amount of radioactivity in the organ of interest at the end of experiment and $AUC_{t_0-t_i}$ is the area under the curve over the time interval t_0 = administration of the compound and t_i = end of the experiment.[20] In studies with ^{125}I-labeled proteins, AUC of TCA-precipitable radioactivity was employed to estimate tissue uptake clearances.

Results

Characteristics of BSA Derivatives—Chemical structures of the radiolabeled proteins are depicted in Figure 1. Some physicochemical characteristics of BSA, Gal-BSA, and Cat-BSA are given in Table 1. The amount of galactose residues attached to BSA was estimated to be 42:1 (mol/mol). The BSA derivatives had nearly the same molecular weights as the original BSA as judged by gel-filtration chromatography. The isoelectric point of Cat-BSA indicated a highly positive charge caused by introduction of hexamethylenediamine into the BSA molecule.

Plasma Profile of ^{111}In and ^{125}I Radioactivity—The time-courses of the radioactivity in plasma and organ accumulation of radioactivity after injection of ^{111}In-DTPA, ^{111}In-SCN-BZ-EDTA, and ^{125}I-labeled compounds into rats are shown in Figure 2. Fast disappearance of radioactivity from the circulation was observed in all experiments and may be accounted for by the avid hepatic uptake of investigated proteins. In the case of Gal-BSA, the initial phase of plasma concentration did not seem to be influenced by the labeling method. However, in the later phase, ^{125}I radioactivity started to increase reaching a peak at 0.75% dose/mL at 60 min followed by a gradual decrease. In the case of Cat-BSA, on the other hand, the distribution patterns varied significantly depending on the labeling methods (see Table 2). Similarly, the latter phase of ^{125}I radioactivity in the plasma was influenced by the reappearance of a ^{125}I-metabolite in the circulation. This, however, did not result in the appearance of a peak. Kidney and spleen showed generally low radioactivity in the Cat-BSA studies and only negligible amounts were found in these organs in the Gal-BSA studies, 3 h after injection.

Biliary and Urinary Excretion of ^{111}In and ^{125}I Radioactivity—Statistically different features of urinary excretion of radioactivity were observed as far as the three methods of labeling are concerned (Figure 3). In the case of ^{111}In labeling, the total radioactivity excreted in the urine within the 180 min of the experimental period was less than 1% of the administered dose, regardless of the BCA used for labeling and regardless of the BSA derivative. On the other hand, the cumulative amount of ^{125}I radioactivity in the urine was 7.8% and 2.9% of the administered dose for Cat-BSA and Gal-BSA, respectively.

The biliary excretion of radioactivity after administration of radiolabeled Cat-BSA was highest for ^{125}I (2.2% of the administered dose) followed by ^{111}In-SCN-BZ-EDTA (1.1% of the dose) and ^{111}In-DTPA (0.3% of the dose). In the case of Gal-BSA, similarly, the radioactivity excretion of ^{111}In-DTPA-labeled compound was the lowest of all, representing 1.7% of the injected dose. There was no statistically significant difference in the biliary excretion of radioactivity of ^{111}In-SCN-BZ-EDTA and ^{125}I-labeled Gal-BSA (3.8% and 3.5% of the administered amount, respectively).

When the biliary excretion rate (BER) of radioactivity was investigated, differences in the rate of appearance of radioactivity in the bile were observed relating to the three methods of labeling (Figure 4). In particular, ^{125}I-bound radioactivity showed a rapid fall in BER after reaching a peak at 25 min. On the contrary, the rate of biliary excretion of ^{111}In-SCN-BZ-EDTA radioactivity, after reaching a maximum, was found to remain constant (Cat-BSA) or only fall gradually (Gal-BSA). The BER value of ^{111}In-DTPA radioactivity (BER_{DTPA}) during the last hour of the experimental period (calculated as the mean of the last three values) was significantly ($p < 0.001$) lower than that of ^{111}In-SCN-BZ-EDTA. The ratio BER_{DTPA}/BER_{EDTA} was 0.20 in the case of Cat-BSA and nearly identical for Gal-BSA (0.19).

Radioactivity Disposition of ^{125}I-Labeled BSA Derivatives and Their Metabolite(s)—The separation of TCA-precipitable ^{125}I radioactivity is shown in Figure 5. It can be seen that the increase in plasma radioactivity after injection of ^{125}I-Gal-BSA was exclusively caused by the appearance of a low molecular weight product in the circulation. Both the urinary and biliary excretion of ^{125}I radioactivity after the administration of ^{125}I-labeled Cat-BSA and Gal-BSA may also be accounted for by the excretion of a degradation product rather than the intact protein. In the urine, no TCA-precipitable radioactivity was detected.

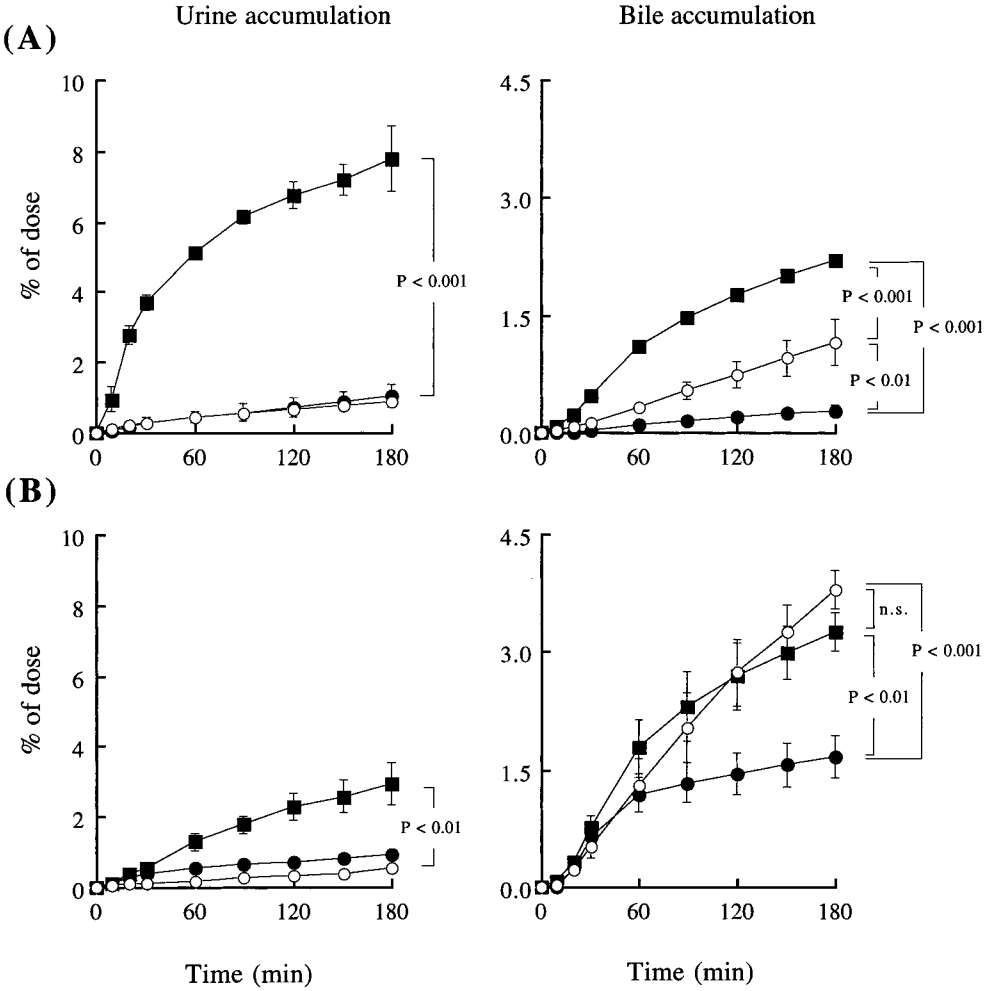

Figure 3—Time-courses of accumulation of radioactivity in the urine and bile after iv administration of Cat-BSA (A) and Gal-BSA (B) labeled with ^{111}In-DTPA (●), ^{111}In-SCN-BZ-EDTA (○), or ^{125}I (■). Data are expressed as mean ± SD of at least three experiments. Statistical significance was based on Student *t*-test; n.s. = statistically nonsignificant.

Apparent Organ Uptake Clearance after Intravenous Administration of BSA Derivatives Labeled with ^{111}In-DTPA, ^{111}In-SCN-BZ-EDTA, and ^{125}I—The apparent volumes of distribution, areas under the radioactivity concentration curve (AUC) and apparent organ uptake clearance of radiolabeled proteins after administration of the investigated compounds in rats are given in Table 2. The AUC of total radioactivity after administration of Cat-BSA was not significantly affected by the method of radiolabeling. However, in the case of Gal-BSA, a rapid release of ^{125}I-metabolite occurred (Figure 2), which resulted in significantly ($p < 0.001$) increased AUC of total radioactivity. When recalculated to infinity, the AUC of ^{125}I radioactivity was about 25 times larger than that of ^{111}In-labeled protein.

The apparent organ uptake clearance of ^{111}In-labeled compounds (regardless of the BCA employed) was consistent with the well-known mechanism of their hepatic uptake. Therefore, the CL_{liver} of ^{111}In-labeled Gal-BSA was very high, approaching the value of liver plasma flow (390 mL/h[21]) ^{111}In−Cat-BSA, on the other hand, was taken up by the liver at a significantly lower rate ($p < 0.001$), with CL_{liver} reaching only about 6% that of Gal-BSA. When ^{125}I was employed as a radiolabel, the apparent hepatic uptake clearance was statistically lower ($p < 0.001$) for both substances, representing approximately 35% of ^{111}In-labeled Cat-BSA. In the case of Gal-BSA, this difference was still more pronounced; the apparent hepatic uptake clearance of ^{125}I-labeled Gal-BSA was less than 10% that of ^{111}In-labeled Gal-BSA.

Statistically lower uptake and/or retention of ^{125}I radioactivity, when compared with ^{111}In, was also observed in the kidney. Uptake of radioactivity by the spleen was observed only for cationized proteins (2−3% of the administered dose), and only a negligible amount of radioactivity was detected in the spleen for the galactosylated proteins.

Time-Course of Organ Distribution of ^{125}I-Labeled BSA Derivatives—The time-courses of ^{125}I radioactivity distribution in the liver, kidney, spleen, and thyroid at 5, 20, 60, and 180 min after injection of ^{125}I−Cat-BSA and ^{125}I-Gal-BSA are shown in Figure 6. In both cases the radioactivity recovered in the liver, kidney, and spleen decreased with time. The most marked fall was seen in the liver during the first 60 min after injection. On the other hand, the thyroid showed an increasing tendency to accumulate ^{125}I radioactivity.

Schematic depiction of radioactivity disposition after administration of radiolabeled proteins in rats is shown in Figure 7.

Discussion

In the previous studies[6,22,23] it was pointed out that the radiolabeled metabolites of liver-specific proteins are the most important determinants of radioactivity elimination

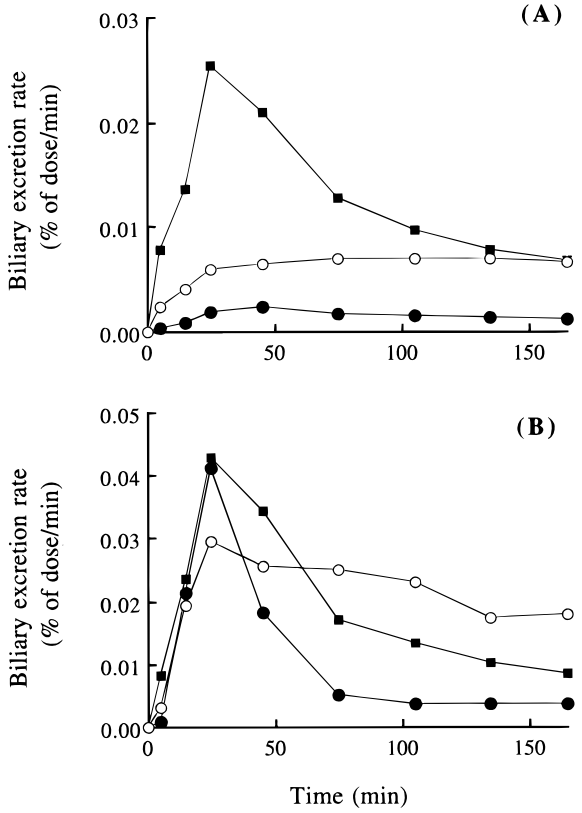

Figure 4—Biliary excretion rate of radioactivity after intravenous administration of radiolabeled Cat-BSA (A) and Gal-BSA (B) in rats. (●) ^{111}In-DTPA; (○) ^{111}In-SCN-BZ-EDTA; (■) ^{125}I. Data represent mean of three rats.

from the target site. Therefore, the choice of an appropriate radionuclide for protein labeling may be the critical step in studies where the body distribution of proteins is investigated and the interpretation of the obtained results is based on radioactivity measurements in various body compartments.

In the present study, we employed two radionuclides, ^{111}In and ^{125}I, to investigate the effect of the radioisotope on the whole body disposition of radioactivity. ^{125}I was attached to the tyrosine residues of proteins using the chloramine T method, and ^{111}In was incorporated into the protein using DTPA or SCN-BZ-EDTA as bifunctional chelating agents. Using these methods, two derivatives of BSA were radiolabeled, and the distribution and excretion of radioactivity was investigated. Gal-BSA and Cat-BSA were chosen as marker compounds because they are known to be taken up by the liver cells via receptor-mediated endocytosis and adsorptive endocytosis, respectively. Previously, we reported the different pharmacokinetics of liver uptake of these two derivatives in rat liver perfusion experiments.[24] Gal-BSA was found to enter the hepatocytes in a dose-dependent (nonlinear) manner with a very fast internalization rate. On the other hand, Cat-BSA was taken up by the liver cells more slowly (internalization constant was about 15% that of Gal-BSA) with a high capacity and was linear over a wide range of inflow concentrations.[24] Consistent results were also obtained in the present study. Both proteins were taken up by the liver in accordance with the type of chemical modification. The CL_{liver} of ^{111}In-labeled Gal-BSA was close to the hepatic plasma flow rate (390 mL/h[21]) and seemed to be limited by this value. On the other hand, the apparent CL_{liver} of ^{111}In-labeled Cat-BSA was significantly lower, representing about 5% that of Gal-BSA.

The fast disappearance of radioactivity from the plasma after intravenous administration of both compounds was caused preferentially by the hepatic uptake of the modified BSAs. In the case of Gal-BSA, there were no significant differences in the distribution phases among the labeling methods. Therefore, it seems plausible that the appearance of the protein in the liver was not influenced by the type of labeling. In the case of Cat-BSA, however, differences between ^{111}In and ^{125}I-labeled proteins were observed. In particular, the distribution half-lives of Cat-BSA labeled with ^{111}In were longer than that of ^{125}I−Cat-BSA (Table 2). We assume that this discrepancy was caused by the anionic character of the chelating agents introduced into the protein molecule. Consequently, the reduced positive charge of the complex resulted in a lower affinity of the protein for adsorptive endocytosis by hepatocytes. This assumption is further supported by the fact that when a 10-fold molar excess of SCN-BZ-EDTA was attached to Cat-BSA, the results obtained from in vivo experiments indicated markedly prolonged retention of radioactivity in the plasma ($t_{1/2\alpha}$ = 13.9 min) with a significantly reduced hepatic uptake clearance (CL_{liver} = 4.74 mL/h) (data not shown).

Significant differences were observed in the extent to which the radiolabel remained in the hepatic tissue. Three hours after drug administration, only a small portion of ^{125}I associated radioactivity was present in the liver (about 5% of the administered dose) when compared with ^{111}In (56−78% dose). Additional distribution experiments with iodine-labeled proteins have revealed a rapid disappearance of ^{125}I radioactivity from the liver within 1 h after drug administration (Figure 6). Then, when the apparent hepatic uptake clearances were calculated, iodinated proteins showed a CL_{liver} that represented only a small fraction of that of ^{111}In-labeled compounds. Therefore, it is evident that the hepatic uptake clearance of these two proteins cannot be simply estimated using ^{125}I radioactivity counts, ignoring the formation of radiolabeled metabolite(s) and radioactivity escaping from the liver. On the other hand, results obtained with ^{111}In-labeled proteins seem to adequately characterize the hepatic uptake of both Gal-BSA and Cat-BSA.

The reason iodine radioactivity escapes from the hepatic tissue much faster than indium radioactivity may be explained in terms of the metabolites that are produced within the hepatic cells. After internalization by the hepatocytes, the proteins are delivered to lysosomes where they undergo proteolytic cleavage and give raise to their metabolic products.[9] In the case of proteins labeled with iodine via tyrosine residues, the metabolites are likely to be mono- and/or diiodinated tyrosine.[10] Either the physicochemical properties or the possible affinity for a transporter system that may be present in the hepatocytes, as speculated by Gore et al.[25] and Duncan and Welch,[22] or a combination of both, enable these metabolites to leave the hepatocytes very easily. In addition, the metabolite may be converted to iodine by cytoplasmic deiodinase[26] and both radiolabeled monoiodtyrosine and iodine were found in blood in studies with ^{125}I-labeled asialofetuin.[27]

In the case of indium-labeled compounds with SCN-BZ-EDTA and DTPA as chelators, ^{111}In-SCN-BZ-EDTA-lysine and ^{111}In-DTPA-lysine were identified as the final metabolic products respectively.[28,23] Slow release of ^{111}In-DTPA-amino acid-associated radioactivity from the liver has previously been reported by Duncan and Welch[22] who pointed out that the compound, to leave the lysosomal compartment, had either to cross the lipid bilayer by simple diffusion or to use a transporter for amino acids. However, simple diffusion is greatly complicated by the ionic character of the chelate and the attached amino acid at intralysosomal pH (about 5.0); carrier-mediated amino acid transport is present for lysine, but the transfer is hindered

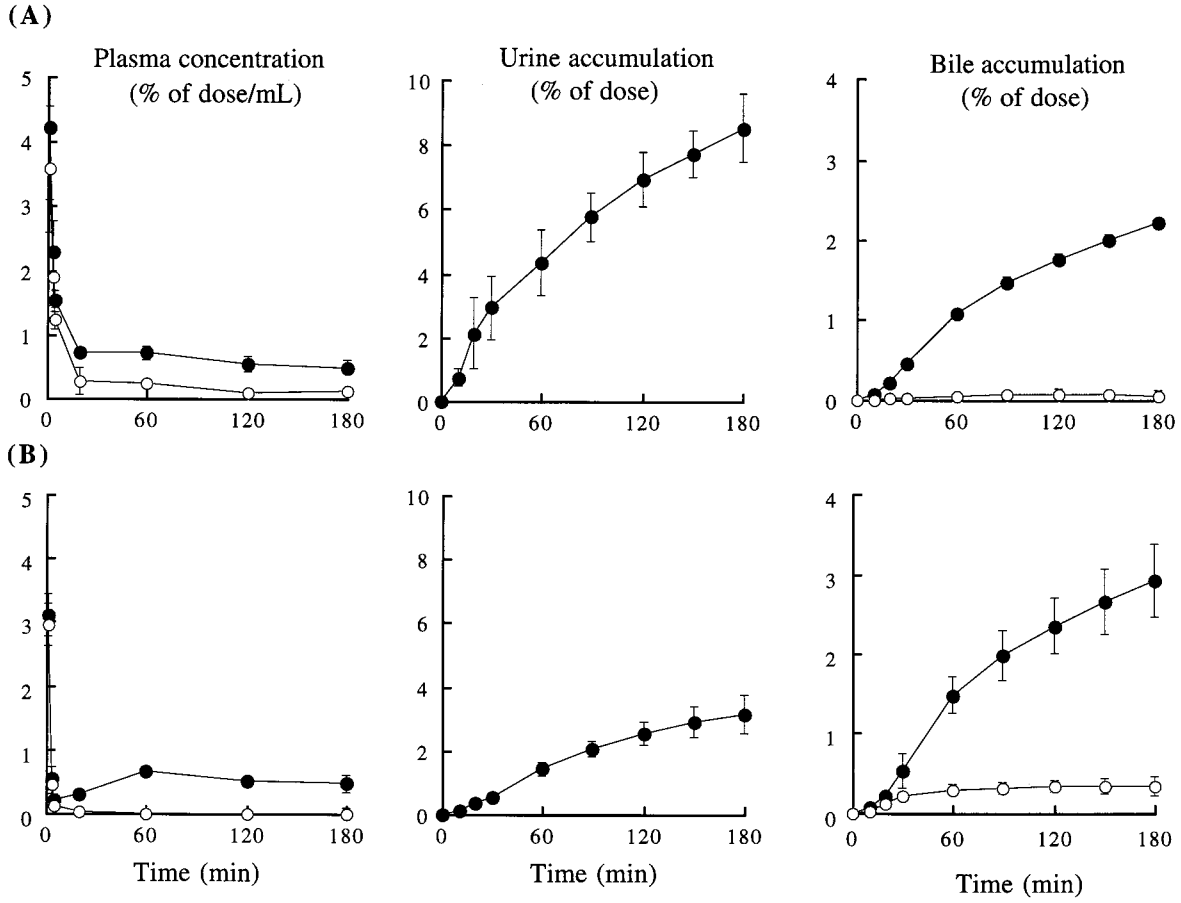

Figure 5—Body disposition of ^{125}I radioactivity; TCA-precipitable (○) and total radioactivity (●) of ^{125}I-labeled Cat-BSA (A) and ^{125}I-labeled Gal-BSA (B). In the urine, no TCA-precipitable radioactivity was detected. Data are expressed as mean ± SD of at least three experiments.

by derivatization of the ϵ-amine. Therefore, the possibility for ^{111}In-DTPA-amino acid to leave lysosomes/hepatocytes is rather limited.

In our study, the different mode and rate of hepatic uptake of both proteins resulted in a different disposition of ^{125}I-associated radioactivity. The internalization and degradation of Gal-BSA take place at a very fast rate. Consequent proteolytic degradation resulted in a rapid escape of radioactivity from the liver (Figure 6) and in the appearance of TCA-soluble ^{125}I radioactivity in the plasma and bile (Figure 5). As a result, the AUC of ^{125}I radioactivity in the plasma was 25 times larger than that of ^{111}In associated radioactivity. A similar course of iodine radioactivity has also been reported in rabbits after administration of ^{131}I-asialoorosomucoid.[29] However, the internalization of Cat-BSA and its delivery to the lysosomal compartment occur at a much slower rate when compared with Gal-BSA,[24] and the production of ^{125}I-metabolite(s) is supposed to be delayed. Therefore, it is unlikely that the fall in the liver radioactivity within 60 min of ^{125}I-Cat-BSA injection and the appearance of TCA-soluble radioactivity in the plasma and urine occurred only due to the formation of a metabolite in the lysosomes. We suppose that it might be caused by an enzymatic degradation (deiodination) of the protein in the plasma, or even when attached to the liver surface, with consequent glomerular filtration of ^{125}I or ^{125}I-radiolabeled metabolite. An attempt to distinguish between the ^{125}I radioactivity associated with protein and that of its degradation product has confirmed that the radioactivity in the urine was accounted for by a metabolite rather than an intact protein (Figure 5). However, the precise structure of the metabolite(s) has not been determined, and therefore we cannot speculate on the exact origin and pathways of the metabolite(s). Nevertheless, when compared with ^{111}In-labeled compounds, ^{125}I labeling resulted in a significantly higher accumulation of radioactivity in the urine in studies with both proteins. In addition, bifunctional chelating agents employed for attaching ^{111}In to protein molecules did not appear to affect the urinary excretion of ^{111}In radioactivity to a great extent.

When the biliary excretion rate was investigated (Figure 4), in the case of Gal-BSA, the curves of BER during the first 25 min followed nearly identical courses. As more than 90% of ^{125}I radioactivity in the bile was TCA-precipitable within 20 min of injection, we assume that during this period the protein avoided the lysosomal compartment and the radioactivity was excreted into the bile in protein-bound form, by means of transcytosis. In a different set of experiments, the gel separation of protein and metabolite-bound radioactivity in the bile in studies with ^{111}In-DTPA labeled Gal-BSA was performed. Similarly, it was found that 80−95% of radioactivity released into the bile was in protein-bound form within ca. 60 min after administration (unpublished results). After this interval, degradation of the protein in the lysosomal compartment occurred and the radiolabeled metabolites were leaving the hepatocytes at different rates in the following order: ^{125}I > ^{111}In-SCN-BZ-EDTA > ^{111}In-DTPA. This escape was in a good agreement with the theory of Duncan and Welch[22] described above. Similar patterns were also observed in the case of Cat-BSA. Interestingly, it was found that the ratio of BERs between ^{111}In-DTPA and ^{111}In-SCN-BZ-EDTA-associated radioactivity was nearly identical for both protein derivatives. Consequently, the cumulative amount of radioactivity in the bile during the 180 min experimental period has shown that, among the three methods of labeling

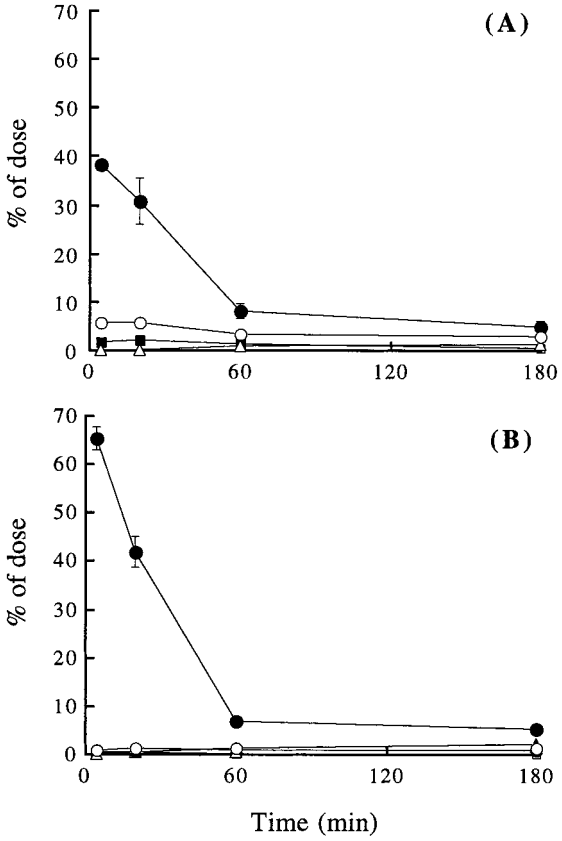

Figure 6—Time-courses of ^{125}I radioactivity distribution in rats after intravenous administration of ^{125}I-labeled Cat-BSA (A) and ^{125}I-labeled Gal-BSA (B). (●) liver, (○) kidney, (■) spleen, (△) thyroid. Data are expressed as mean ± SD of two experiments.

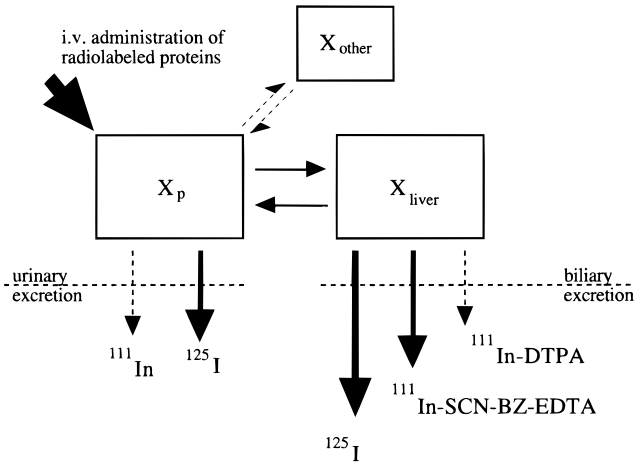

Figure 7—Schematic depiction of radioactivity disposition after intravenous administration of radiolabeled liver-specific protein in rat. X_p = radioactivity in the plasma; X_{liver} = radioactivity in the liver; X_{other} = radioactivity in other tissue.

employed, ^{111}In-DTPA-associated radioactivity appeared in the bile in the lowest amount, regardless of the protein derivative. ^{111}In-SCN-BZ-EDTA radioactivity was excreted into the bile in a higher amount than that of ^{111}In-DTPA. This is consistent with the previous results of Arano et al.[30] who reported faster biliary elimination of ^{111}In-SCN-BZ-EDTA-lysine in comparison with ^{111}In-DTPA-lysine. Also, a similar set of experiments performed in mice revealed comparable behavior of these radiolabels. However, the difference between ^{111}In-DTPA and ^{111}In-SCN-BZ-EDTA radioactivity was much more pronounced.[31] The faster elimination of ^{111}In-SCN-BZ-EDTA-labeled metabolite might also be explained in terms of the physicochemical properties of the radiometabolites. However, the detailed mechanism of the disappearance of these compounds from the liver cells remains to be elucidated.

For a more accurate estimation of the CL_{liver} of ^{125}I-labeled proteins, the more sophisticated approach of a pharmacokinetic analysis has been adopted in our previous study in mice.[31] TCA-precipitable fraction was employed for data analysis and the time-courses of radioactivity in the plasma and liver were simultaneously fitted to exponential equations to calculate the CL_{liver}. However, even this approach, requiring much more experimental data, did not produce satisfactory results due to the pattern of liver accumulation.

In conclusion, this study has confirmed a significant influence of the radioisotope employed in the labeling of the liver-specific proteins on the fate of the radioactivity in the body. ^{125}I radioactivity rapidly escaped from the target tissue and was excreted into bile and urine, thus interfering with the pharmacokinetic analysis to a great extent. Therefore, ^{125}I labeling was found inappropriate for distribution studies of Gal-BSA and Cat-BSA in rats. On the other hand, the results obtained with ^{111}In were consistent with the well-known behavior of these proteins in the organism and allowed pharmacokinetic analysis to be performed without correction for radiolabeled metabolites. In particular, because of the restricted release of ^{111}In-DTPA-labeled metabolite from the liver, among the three methods of radiolabeling investigated, ^{111}In radiolabeling using DTPA as a bifunctional chelating agent was found to be the most suitable for in vivo disposition studies of liver-specific proteins.

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JS9804415