A Facile Method for the Labeling of Proteins with Zirconium Isotopes

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ABSTRACT. To label proteins with positron emitters with a half-life in the order of days, a method has been developed to label proteins with zirconium (Zr)-isotopes. Therefore, the bifunctional chelating agent desferal (Df) was coupled to albumins via a thioether bond. Labeling of the premodified proteins was easily performed by addition of these proteins to freeze-dried Zr-oxalate. This labeling was efficient (>90%) and accomplished in several minutes. The conjugates showed a high in vitro stability. Biodistribution studies were performed with ^{88}Zr-citrate, ^{88}Zr-Df, and ^{88}Zr-labeled mouse serum albumin (^{88}Zr-Df-MSA), modified with different amounts of chelating groups. Whereas Zr-citrate was found to accumulate in bone, Zr-Df was cleared very fast by glomerular filtration. The ^{88}Zr-Df-MSA showed similar blood clearance as did ^{123}I-labeled MSA. The biodistribution pattern of ^{88}Zr-Df-MSA differed only from ^{123}I-MSA in that a higher accumulation of Zr in liver, kidney, and spleen was found. The absence of large amounts of ^{88}Zr in bone indicated that in vivo the conjugates are also reasonably stable. NUCL MED BIOL 23;4:439–448, 1996.

INTRODUCTION

Positron emission tomography (PET) is a noninvasive imaging technique that provides quantitative data on the biodistribution of radiolabeled compounds. A method to label proteins with positron emitting nuclides might be valuable, considering the ubiquitousness of proteins and peptides and their role in many disease processes. To be useful, the positron emitter must fulfill several requirements. Its production must be simple and inexpensive, and it should not give off radionuclidic impurities. The radionuclide should by preference have a high percentage of positron emission as the only form of decay.

Proteins have been labeled with several positron-emitting isotopes such as ^{18}F ($t_{1/2}$ = 110 min; 7, 13, 31), ^{68}Ga ($t_{1/2}$ = 68 min; 31), ^{64}Cu ($t_{1/2}$ = 12.7 h; 20, 25, 32), and ^{55}Co ($t_{1/2}$ = 17.5 h; 22, 37). However, the short half-lives of these isotopes hamper the use for the long-term determination of the kinetics and biodistribution of proteins. For instance, they are less suitable for quantitating the biodistribution of monoclonal antibodies (MAb) in order to generate accurate dosimetric data necessary for radioimmunotherapy. The positron emitter ^{89}Zr ($t_{1/2}$ = 78.4 h) has an appropriate half-life. Although this positron emitter does not fulfill all prerequisites—for instance, it decays for only 23% by positron emission and has accompanying γ-radiation—the suitable half-life justifies the choice for this isotope. ^{89}Zr can be produced on ^{89}Y (100% natural abundance, 3, 18, 41) in high yield and a high (radio)chemical purity (24). Although ^{89}Zr seems to be a suitable candidate, until now

little effort has been made to label proteins with this isotope. Here we describe the development of a simple and efficient method to label proteins with Zr-isotopes, using albumins as model.

MATERIALS AND METHODS
Materials

Desferal (Df) was obtained from Ciba-Geigy (Basel, Switzerland). N-succinimidyl S-acetylthioacetate (SATA), succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), and iodogen were obtained from Pierce (Rockford, IL). Oxalic acid, sodium hydroxide, hydroxylamine, sodium citrate, and sodium phosphate ($Na_2HPO_4 \cdot 12H_2O$ and $NaH_2PO_4 \cdot H_2O$) were from Merck (Darmstadt, Germany). Ammonium acetate, n-butanol, hydrochloric acid, acetic acid, and sodium azide were obtained from J. T. Baker Chemicals BV (Deventer, The Netherlands) and iron(III)-chloride was from Aldrich (Borhem, Belgium). Bovine serum albumin (BSA), mouse serum albumin (MSA), and 5,5'-dithio-bis-2-nitrobenzoic acid (Ellman's reagent) were from Sigma (St. Louis, MO) and mercaptoethanol was from LKB (Bromma, Sweden). BALB/c mice were obtained from Harlan CPB (Zeist, The Netherlands). The ^{88}Zr, used as tracer instead of ^{89}Zr because of its more convenient half-life ($t_{1/2}$ = 83.4 days), was produced as described by Meijs et al. (24). The ^{123}I was produced by the (p,2n) reaction on 99.98%-enriched ^{124}Xe (^{124}Xe(p,2n)^{123}Cs → ^{123}Xe → ^{123}I). Ultrapure water was used throughout the experiments to minimize the amount of metal impurities in the solutions.

General Procedures

After labeling, the proteins were analyzed by instant thin layer chromatography (ITLC) and thin layer chromatography (TLC).

The ITLC was performed on silica-gel-impregnated glass fiber sheets (Gelman Sciences Inc., Ann Arbor, MI) with 0.5 M sodium citrate (pH = 4.5) as mobile phase. The TLC was performed on aluminum-backed cellulose plates (Merck, Darmstadt, Germany); *n*-butanol/water/acetic acid (2/1/1) was used as mobile phase. After elution, the plates were cut into 10 pieces and assayed for radioactivity using a 1282 Compugamma counter (Wallac, Turku, Finland).

Gel filtration was performed on G-25 (PD-10-columns) and G-50 using a 0.1 M acetate buffer (pH = 6) as eluent, unless described otherwise. The G-25 and G-50 gel filtration column materials were from Pharmacia Biotech (Roosendaal, The Netherlands).

High-performance liquid chromatography (HPLC) was performed on a Protein Pak 300 SW column (Waters, Milford, MA) using a Pharmacia HPLC-pump (2150). Ammonium acetate (0.2 M, pH = 6.5) containing 0.05% sodium azide was used as eluent (flow rate 0.5 mL/min). A UV-detector (Pharmacia, Uppsala, Sweden) at 280 nm and a flow-through radioactivity detector (Ortec 406A single-channel analyzer) were used to monitor the eluate.

"Direct Labeling" of Bovine Serum Albumin with ^{88}Zr (^{88}Zr-BSA)

The ^{88}Zr was isolated in oxalic acid with a hydroxamate-column as described by Meijs et al. (24). The oxalic acid was removed by sublimation at room temperature *in vacuo*. BSA (100 μg) in 100 μL of either 0.1 M acetate buffer (pH = 5 and 6) or 0.1 M citrate buffer (pH = 5 and 6) was added to this freeze-dried Zr-oxalate. After 1 h of incubation the protein was analyzed by G-50 gel filtration and ITLC.

N-(S-acetyl)Mercaptoacetyldesferal (SATA-Df)

SATA-Df (Scheme 1) was prepared by dissolving Df (0.473 mmol) in 10 mL of water. The pH was adjusted to 9 using 1 M NaOH. SATA (0.179 mmol) was added and the reaction mixture was allowed to stand at room temperature for 2 h. Hereafter, the solution was acidified to pH = 2 using concentrated HCl, resulting in precipitation of SATA-Df, while unreacted Df remained in the solution. Recrystallization from 10 mL of 1 mM HCl yielded 0.139 mmol SATA-Df (78%). The product was analyzed by TLC and mass spectrometry (Finnigan MAT90, Bremen, Germany). Silicaplates (DC Alufolien from Merck, Darmstadt, Germany) were used for the TLC analysis. Spots were eluted with MeOH and detected by spraying with 1% FeCl$_3$; the product was found to have an Rf-value of 0.65, while Df remained at the origin. MS-results: (Df-NH-CH$_2$-S-C(O)-CH$_3$)H$^+$ = 677 (4%), (Df-NH-CH$_2$-SH)H$^+$ = 635 (40%) and (Df-NH-CH$_3$)H$^+$ = 603 (100%).

Preparation of Albumin-Desferal Conjugates (BSA-Df)

To 2 mL of BSA solution (20 mg, 300 nmol) in phosphate buffer (pH = 8.5, 0.1 M) 20 μL of a freshly prepared SMCC solution in DMF (150 mM, 3 μmol) was added. After incubation for 30 min the protein was separated from the excess of SMCC by gel filtration using a PD-10 column pre-eluted with 0.1 M phosphate buffer (pH = 6.5). To the modified protein, 20 μL of a freshly prepared SATA-Df solution in DMSO (150 mM, 3 μmol) and 20 μL of freshly prepared hydroxylamine in phosphate pH = 6.5 (50 mg/mL, 30 μmol) were added. After 1 h at room temperature the excess of SATA-Df and hydroxylamine was removed with G-50 gel filtration.
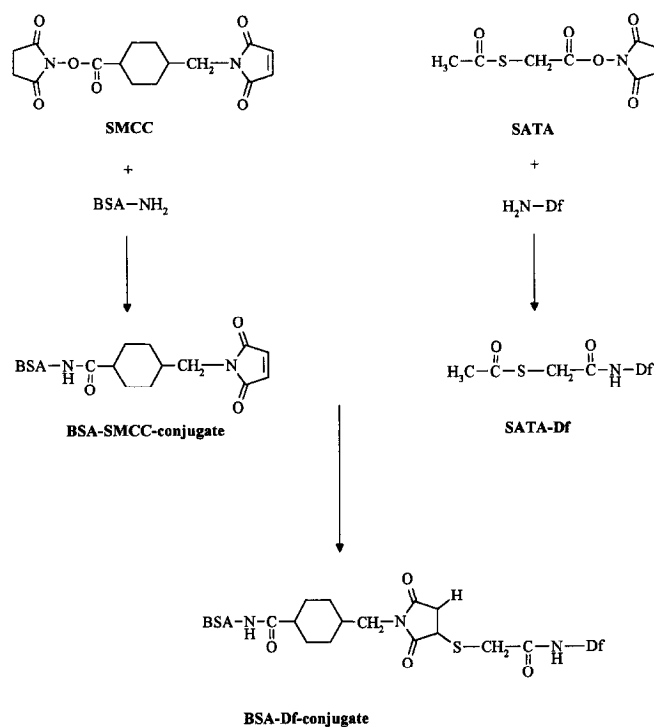
SCHEME 1

The number of maleimide groups in the protein (before reaction with SATA-Df) was determined chemically using Ellman's reagent. For this purpose, 50 μL of mercaptoethanol (96.5 nmol) was added to 50 μL of reaction mixture and diluted with 1.5 mL of phosphate buffer (0.1 M, pH = 8.5). Finally, 50 μL of Ellman's reagent (4 mg/mL) was added. After 15 min of incubation at room temperature the UV-absorbance was measured at 412 nm. Standards of mercaptoethanol from 0 to 100 nmol were used for quantification. The protein concentration was determined using biorad protein assay (München, Germany) with BSA solutions (0–0.5 mg/mL) as standards.

Molecular-weight measurements were performed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) using a Finnigan MAT Vision 200 laser desorption time-of-flight mass spectrometer (12).

Labeling of BSA-Df with ^{88}Zr (^{88}Zr-Df-BSA)

The BSA-Df in 100 μL of acetate (0.1 M, pH = 6) was added to freeze-dried ^{88}Zr-oxalate (*vide supra*). The reaction mixture was analyzed by gel filtration (G-25, G-50, and HPLC) and ITLC. To optimize the labeling, the conditions were varied and the reaction mixtures were analyzed with ITLC. The time dependency of the labeling efficiency between 5 min and 2 h was examined. The influence of the protein concentration was determined using BSA-Df at 3, 1, 0.5, 0.1, and 0.05 mg/mL. The labeling efficiency was determined after 1 h of incubation at room temperature.

In Vitro Stability Studies

The difference in stability of the protein-conjugate (0.42 mg/mL) obtained both by "direct" labeling or via Df was determined in citrate buffer of different concentrations (0.167, 1.67, 16.7, 167, and

1670 mM). The percentage of ^{88}Zr attached to the protein was measured using gel filtration.

The *in vitro* stability of the Zr-Df-conjugate was measured by incubation ^{88}Zr-Df (180 μM) or ^{88}Zr-Df-BSA (0.8 mg/mL) in water, 0.8 mg/mL BSA, 20% human serum and 0.8 mM citrate and subsequent analysis with TLC.

Labeling of MSA with ^{88}Zr

MSA was used to investigate the *in vivo* behavior of ^{88}Zr-labeled albumin and to investigate the dependence of the biodistribution on the modification grade. For these purposes MSA was modified in accordance with the BSA modification, but with different SMCC/MSA ratio (1.5, 3, 6, and 10, respectively). Labeling was performed for 1 h at protein concentrations of 3 mg/mL to maximize the yield, which were found to be above 90%.

Animal Distribution Measurements

Animal experiments were carried out in accordance with the recommendations of the National Council on Animal Care and of the in-house Ethics Committee. Three different protocols were performed with female BALB/c mice of 6–7 weeks. When the animals were under anesthesia, labeled compounds (100 μL in 0.1 M acetate, pH = 7.0) were administered in the retro-ocular plexus. At various intervals, mice were sacrificed and dissected. Tissues of interest were removed, weighed, and assayed for radioactivity. The percentage injected dose per gram tissue (%ID/g) was calculated. Furthermore, the urine at the moment of sacrifice was collected and assayed for radioactivity.

To determine the biodistribution of free Zr, sodium citrate (0.4 mM) was added to freeze-dried ^{88}Zr-oxalate (*vide supra*). The ^{88}Zr solution (14.4 μCi) was injected and the tissue distribution was determined at 5 min, 4 h, and 24 h. The biodistribution of Zr-Df was determined as follows: A Df solution (10 mM in acetate) was added to freeze-dried ^{88}Zr-oxalate. After dilution to 0.75 mM Df, 12.3 μCi of ^{88}Zr-Df complex was injected, and after 5, 15, and 30 min the biodistribution was determined. Biodistribution of the different ^{88}Zr-Df-MSA conjugates (*vide supra*) was examined by injection of about 7–8 μCi (1–1.5 μg). After 1, 4, and 24 h the biodistribution was determined. As a control we also examined the biodistribution of ^{123}I-labeled MSA (94 μCi, 8.7 μg), labeled with the use of iodogen as described by Haisma *et al.* (8), at the same time-points in a parallel study.

RESULTS
"Direct Labelling" of BSA with ^{88}Zr

In the presence of 0.1 M acetate there was a variable amount (30–90%) of ^{88}Zr co-migrating with BSA when analyzed by G-50 gel filtration (Fig. 1a). Besides the binding to the protein, a variable amount of ^{88}Zr remained associated to the column. In the presence of 0.1 M citrate, no "direct labeling" of the protein and hardly any association to the column were observed. ITLC analysis of the reaction mixtures, in the presence of both acetate and citrate, showed little or no ^{88}Zr associated with the protein (Fig. 1b).

Conjugation Chemistry and Labeling

SATA-Df was synthesized in high yield (78%). Maleimide groups were coupled to the albumin with SMCC (Scheme 1). The cou-

pling yield was 50–60% (Ellman's reagent and mass spectroscopy, MS), which corresponded with five to six maleimide groups per albumin. About 10–20% of these maleimide groups reacted with SATA-Df as determined with MS, resulting in 0.5–1 Df groups per protein. The remaining maleimide groups reacted with the excess of hydroxylamine, preventing further reaction with SH groups. This reduced the possibility for protein aggregate formation. During the modification steps there was no alteration in the HPLC pattern when compared to native albumin.

The labeling was performed by addition of the premodified albumin to freeze-dried ^{88}Zr-oxalate. It was found that at increasing concentration of BSA-Df the labeling efficiency increased (Fig. 2). Under standard labeling conditions (1 mg/mL, 1 h) the ITLC and G-50 analyses (Fig. 1c,d) demonstrated a labeling efficiency of more than 90%. The HPLC pattern of the reaction mixture showed that all radioactivity was co-migrated with the native albumin. To be sure of an efficient labeling, a labeling time of 1 h was used in further experiments, although it was found in the optimizing experiments that after 5 min the labeling efficiency did not increase (at protein concentration of 1 mg/mL).

Stability Studies

Above citrate concentrations of 1.6 mM, Zr is transchelated from the "direct labeled" BSA (Zr-BSA) to citrate (Fig. 3). In the case of Zr-Df-BSA, hardly any Zr-citrate was formed even at high citrate concentrations (167 mM), demonstrating that the binding of ^{88}Zr via the bifunctional chelating agent Df is more stable toward citrate than that of directly bound ^{88}Zr.

The Zr-Df complex was incubated in several solutions of interest: citrate, BSA solution, and human serum, and subsequently the amount of Zr present in the Zr-Df complex was measured using TLC. In all solutions less than 0.5% of the Zr was lost from the complex over 24 h.

The stability of the binding between Df and the protein was investigated by storage of ^{88}Zr-Df-BSA in the above-mentioned solutions and subsequent determination of the amount of ^{88}Zr-Df using TLC. Over a 10-day period no loss of ^{88}Zr-Df occurred in all solutions, indicating that the thioether binding between Df and BSA is stable *in vitro*.

Biodistribution

For a better understanding of the *in vivo* behavior of Zr-labeled proteins, we also examined the biodistribution of ^{88}Zr-citrate and ^{88}Zr-Df. The blood clearance of the ^{88}Zr-compounds was dependent on the chemical form of the ^{88}Zr (Fig. 4). Zr-Df was cleared quickly from the circulation. After 5 min more than 23% of the injected dose was found in the urine. This fast glomerular excretion probably caused the temporary high kidney level. Uptake in other organs did not exceed 2% after 30 min (Table 1). The clearance of ^{88}Zr-citrate was slower than that of the Zr-Df complexes; after 4 and 24 h, 16.3 and 3.0 %ID/g, respectively, were still present in the blood. The Zr was finally deposited in bone (21 %ID/g at 24 h). The levels of Zr present in liver and kidney did not follow the clearance of the blood (Fig. 4), suggesting that Zr in these organs might be present in the intracellular species.

The biodistributions of ^{88}Zr-Df-MSA and ^{123}I-MSA are depicted in Fig. 5. The blood clearance of these conjugates was similar and significantly slower than those of Zr-Df and Zr-citrate (Fig. 4). The biodistribution of ^{88}Zr-Df-MSA resembled that of ^{123}I-MSA,
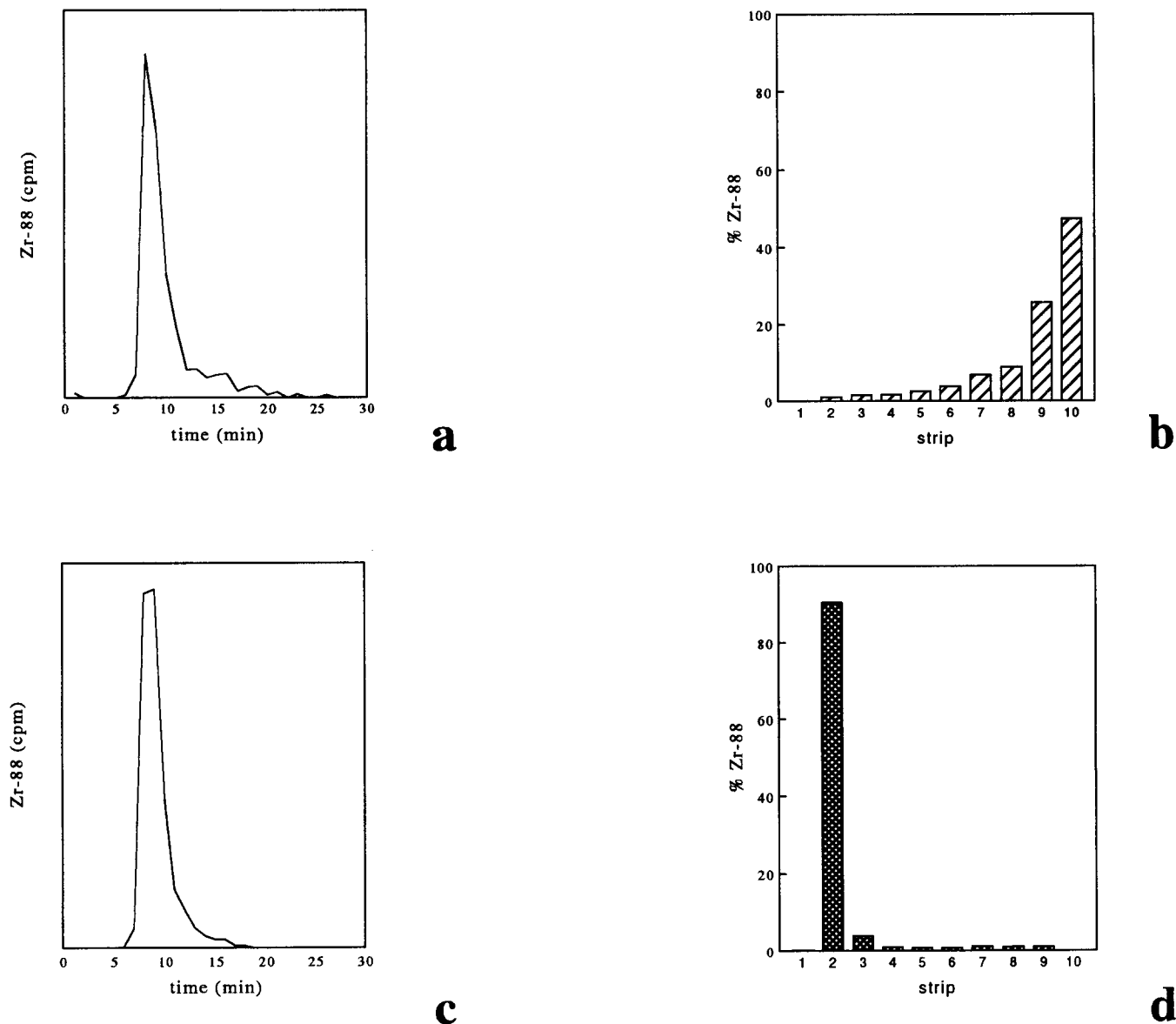
FIG. 1. The gel filtration profile and corresponding ITLC-pattern of BSA labeled with ^{88}Zr with the "direct" method (a,b) and via desferal (c,d).

except for the uptake in liver, spleen, and kidneys, which was higher for ^{88}Zr-Df-MSA. The amount of ^{88}Zr in these organs remained the same over the time period measured, whereas the amount of ^{123}I decreased in time (Fig. 4). At higher SMCC/MSA ratio, the liver accumulation is higher and the kidney accumulation is lower (Fig. 6).

DISCUSSION

The physical characteristics of the positron emitter ^{89}Zr make it a good candidate for the labeling of proteins for various applications: for instance, to study the kinetics and biodistribution of proteins over a period of several days. Thus far, little effort has been made for labeling proteins with this isotope. The purpose of our study was to develop an efficient and practical method to label proteins with Zr-isotopes, keeping in mind that the radioconjugate must be stable

in vitro as well as *in vivo* and that the pharmacokinetic behavior must be conserved.

The most simple method for labeling proteins would be a direct labeling, making use of the chelating groups of the protein itself for complexation. Because Zr has a tendency to form complexes with a large variety of chelating agents and because it has been shown that Zr easily transchelates to proteins (18, 21), it seemed worthwhile to investigate such a direct method of labeling. Indeed, the results obtained with gel filtration indicated that simple addition of BSA to ^{88}Zr in the presence of acetate yielded a labeled protein (Fig. 1a). However, these results were not confirmed by ITLC analysis (Fig. 1b). This was thought to be caused by transchelation of Zr from the chelating groups of the protein to the citrate present in the eluent. This assumption was confirmed by the fact that, in the presence of 0.1 M citrate, no labeling of BSA with ^{88}Zr was observed at all. The binding between the radiometal and the protein must be stable;
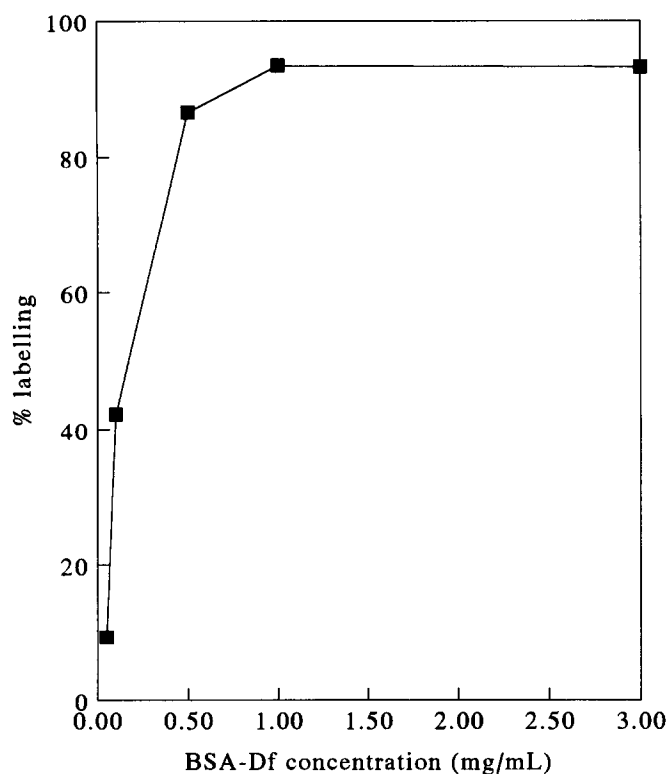
FIG. 2. Efficiency of the labeling as function of the modified protein concentration.

FIG. 3. Transchelation of ^{88}Zr from BSA (labeled with the "direct" method, 0.5 mg/mL, □) and ^{88}Zr-Df-BSA (labeled via desferal, 0.5 mg/mL, ■) to citrate at different concentrations.

therefore, owing to this *in vitro* instability, this direct labeling was rejected as a useful method. These results, however, showed that Zr has a tendency to bind nonspecifically to proteins.

An alternative for the labeling of proteins with Zr-isotopes is the use of a bifunctional chelating agent. DeJesus and Nickels (3) have labeled polylysine with ^{89}Zr, using DTPA. However, in earlier studies we found that Zr-Df complexes are much more stable than Zr-DTPA (23). This high stability of the Zr-Df complexes was confirmed in the stability experiments performed in this study. In the presence of serum, albumin, and citrate, the Zr lost from the Df was less than 0.5% during a 24-h period. Df, a nontoxic agent that is clinically used for reduction of iron plasma levels, has been used as a chelating agent for the labeling of proteins with Ga-isotopes. Incorporation of Df groups into proteins can be performed with the homofunctional agent glutaraldehyde (26, 29, 40) and with heterofunctional agents like carbodiimides (11, 26). Because these agents have the tendency to form protein aggregates, Koizumi *et al.* (14) developed several other methods for coupling. With respect to the *in vivo* stability they found antibody conjugates linked by thioether bonds to be optimal. Such a method was also used by Arano *et al.* (1, 2) to couple Df via a metabolizable linker to antibodies. We have coupled Df to the protein via a thioetherbond. Maleimide groups were coupled to the protein using SMCC and reacted with SATA-Df (Scheme 1). The reaction conditions were mild, and with respect to the native protein no detectable increase in aggregation was observed by HPLC. No loss of Df was observed after several days of storage in different solutions (water, citrate, and albumin). Mass spectroscopy (MS) was used to quantitate the amount of incorporated maleimide and Df groups. The amount of maleimide groups (5–6 per BSA) determined with MS was in agree-
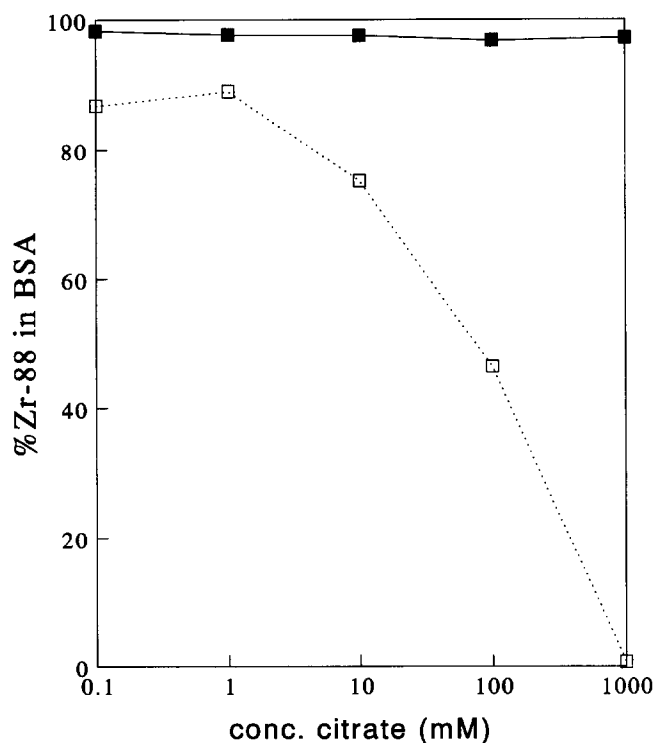
ment with the results found with the chemical method using Ellman's reagent. An advantage of MS is that even samples of only 10 μg can easily be measured.

The labeling of Df-modified proteins with Ga isotopes has been discussed in the literature, but the use of Df to label proteins with Zr has not been described before. Both G-50 and ITLC analyses showed that simple addition of Df-modified protein (BSA-Df) to freeze-dried ^{88}Zr-oxalate resulted in efficient labeling (>90%, Fig. 1c,d). Furthermore, the modified protein can be prepared several weeks before actual use; thus, this method of labeling might be convertible into a kit form. Because labeling with no carrier-added radioactive material follows pseudo first-order kinetics, the yield of labeling is only affected by the concentration of Df groups (Fig. 2).

A suitable method of labeling should not only be efficient and give stable conjugates, but the pharmacokinetical behavior of the protein must also be retained. To investigate whether our method of labeling alters the biodistribution of proteins we used the protein MSA (mouse serum albumin) as a model. This protein was used for its relative inertness in mice and because no immunological reactions were expected. Furthermore, owing to the fact that the amount of labeled MSA injected is negligible with respect to the amount of MSA already present in the mouse, concentration effects can be excluded.

To get an insight in the *in vivo* behavior of the radioconjugate, the biological properties of some degradable fragments such as free ^{88}Zr and ^{88}Zr-Df were determined separately. Zr-Df was cleared quickly from the circulation by the kidneys, a phenomenon that was also observed for Ga-Df complexes (9, 16). ^{88}Zr-citrate was used as source of free Zr; the small amount of citrate with respect to the
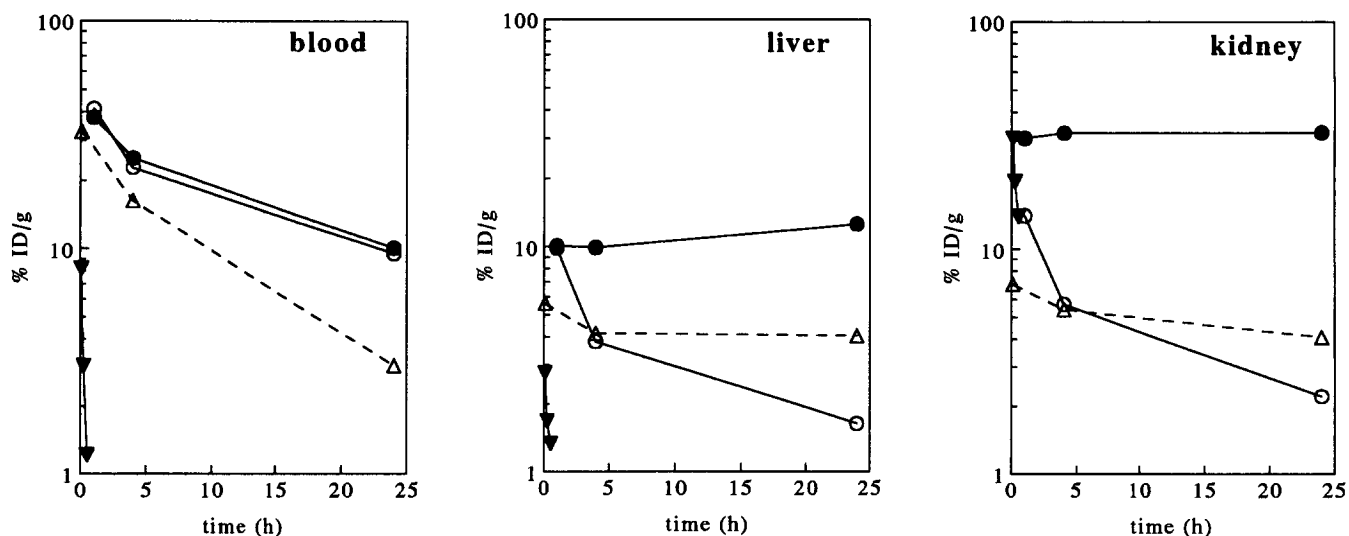
FIG. 4. Pharmacokinetics of ^{88}Zr-Df (—▼—), ^{88}Zr-citrate (—-△—-), ^{88}Zr-Df-MSA (—●—), and ^{123}I-MSA (—○—) in BALB/c mice.

large amount of proteins in the plasma is neglectable. The ^{88}Zr remained longer in the circulation than did ^{88}Zr-Df (Fig. 4) and was finally deposited in the bone (Table 1). The high bone accumulation is in agreement with results found by Shiraishi and Ichikawa (35) and Fletcher (5) and emphasizes the importance of a high stability of the conjugate. The similarity of the blood clearance of ^{88}Zr-Df-MSA and ^{123}I-MSA suggests the same rate of *in vivo* degradation of both conjugates (Figs. 4 and 5). The main differences between the biodistribution of ^{88}Zr-Df-MSA and ^{123}I-MSA are the higher accumulation of ^{88}Zr in the liver and kidneys.

Initially the amounts of ^{123}I and ^{88}Zr present in the liver were the same, but the amount of ^{123}I rapidly decreased, whereas the amount of ^{88}Zr remained more or less constant (Figs. 4 and 5). Fast excretion of iodine from the liver and retention of radiometals in the liver is a well-known phenomena for radiolabeled antibodies (10, 15, 34) and is attributed to trapping of the labeled catabolic products by liver cells (4, 6, 27, 28).

Activity found in the kidneys (±30%ID/g) accounts for only 7% of the injected dose. This relatively high kidney accumulation cannot be due to breakdown of the conjugate, resulting in a release of

^{88}Zr-Df, because this complex is rapidly excreted (Fig. 4). Therefore, it is more likely that the accumulation of Zr in the kidneys is, in analogy with antibody fragments labeled with radiometals (33, 36), the result of albumin degradation in this organ (19). The differences seen for Zr and I-labeled MSA are mainly restricted to a larger amount of Zr in the organs responsible for the protein degradation and a larger amount of iodine present in the stomach. These differences are probably caused by different behavior of the labeled catabolic product and are in accordance with differences in *in vivo* behavior found for In- and Ga-labeled proteins with respect to I-labeled proteins (30, 34). Therefore, it was suggested that the *in vivo* behavior of the albumin was hardly altered as a consequence of the labeling of the protein with ^{88}Zr.

The influence of protein modification was determined by differing the amount of maleimide groups and, subsequently, the amount of Df groups. The larger the amount of modification, the larger the amount of Zr in liver and the lower the kidney accumulation (Fig. 6). A faster blood clearance and increase in liver accumulation as a result of overloading has also been shown for ^{67}Cu and ^{99m}Tc-labeled proteins in animals (17, 38) as well as in man (39). This

TABLE 1. Distribution of ^{88}Zr-Citrate and ^{88}Zr-Df in BALB/c mice (n = 3), represented as %ID/g (SD)

| Tissue	^{88}Zr-citrate			^{88}Zr-desferal		
	5 min	4 h	24 h	5 min	15 min	30 min
Blood	32.88 (5.29)	16.28 (1.01)	3.03 (1.22)	8.18 (1.35)	3.04 (0.92)	1.21 (0.34)
Heart	10.33 (0.83)	5.03 (0.30)	1.75 (0.37)	3.32 (0.51)	1.10 (0.36)	0.60 (0.14)
Liver	5.62 (1.30)	4.14 (0.19)	4.04 (0.31)	2.76 (0.56)	1.68 (0.28)	1.33 (0.15)
Spleen	5.83 (1.49)	3.41 (3.00)	2.83 (0.79)	2.40 (0.66)	1.10 (0.20)	1.04 (0.14)
Kidney	6.99 (1.78)	5.41 (0.87)	4.07 (0.32)	30.70 (3.01)	19.89 (4.82)	13.98 (2.63)
Stomach	3.57 (0.88)	3.37 (0.35)	1.50 (0.09)	3.02 (0.71)	1.28 (0.30)	0.75 (0.24)
Bone	4.12 (0.33)	6.87 (0.77)	20.90 (5.32)	2.01 (0.42)	0.87 (0.25)	0.55 (0.20)
Muscle	1.70 (0.12)	1.74 (0.06)	1.17 (0.03)	1.66 (0.19)	0.77 (0.32)	0.37 (0.19)
Lung	12.07 (1.86)	5.92 (1.43)	3.14 (1.13)	5.31 (0.83)	2.23 (0.52)	1.14 (0.28)
Intestine	3.96 (0.14)	1.66 (0.31)	0.96 (0.31)	1.93 (0.14)	1.32 (0.35)	1.21 (0.92)
Fat	1.30 (0.31)	1.77 (0.64)	0.73 (0.24)	2.09 (1.25)	0.84 (0.23)	0.44 (0.34)
Skin	1.56 (0.72)	4.53 (0.39)	2.34 (0.32)	3.93 (0.40)	2.47 (0.77)	1.21 (0.49)
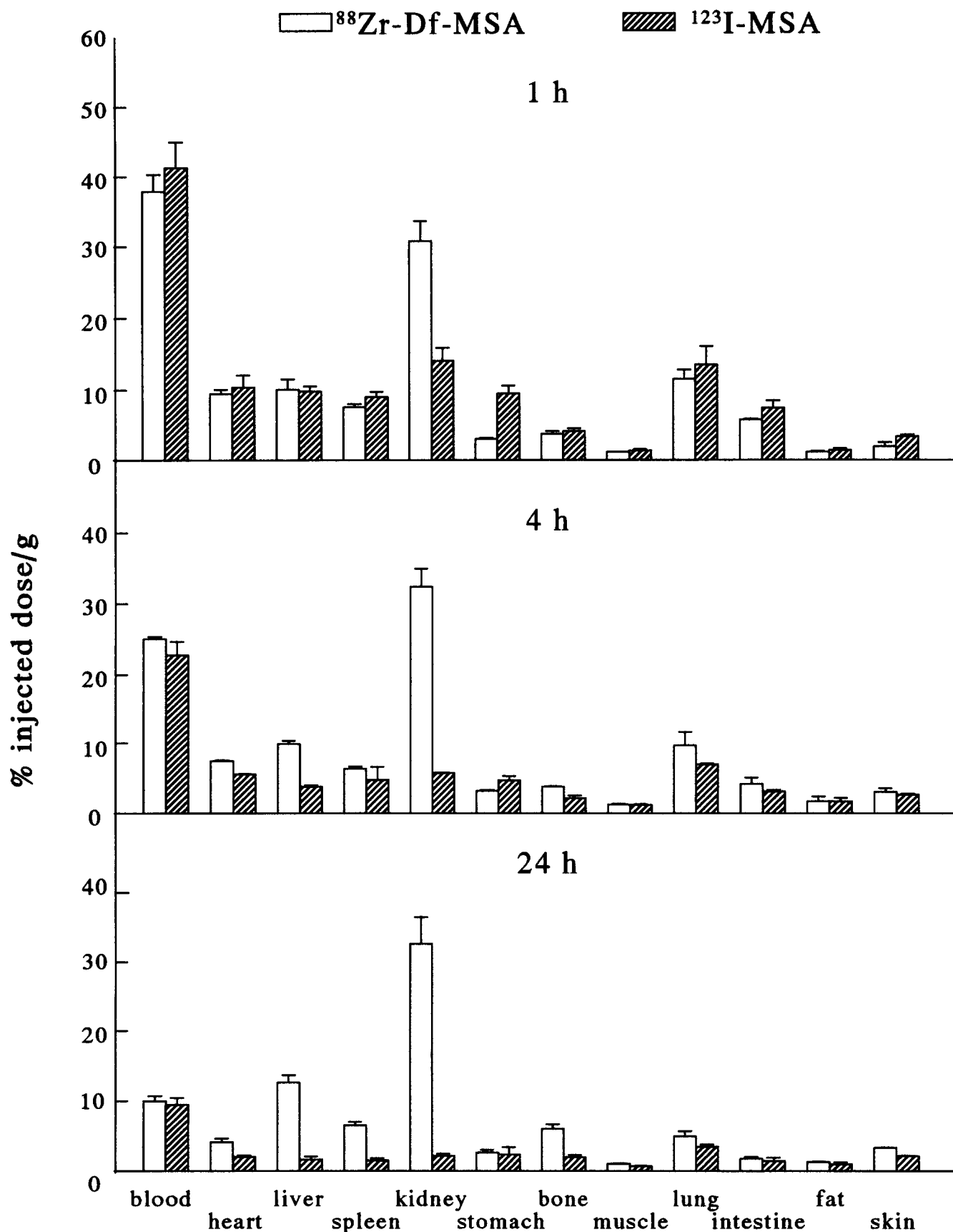
FIG. 5. Biodistribution of [88]Zr-Df-MSA and ^{123}I-MSA in BALB/c mice (n = 3).
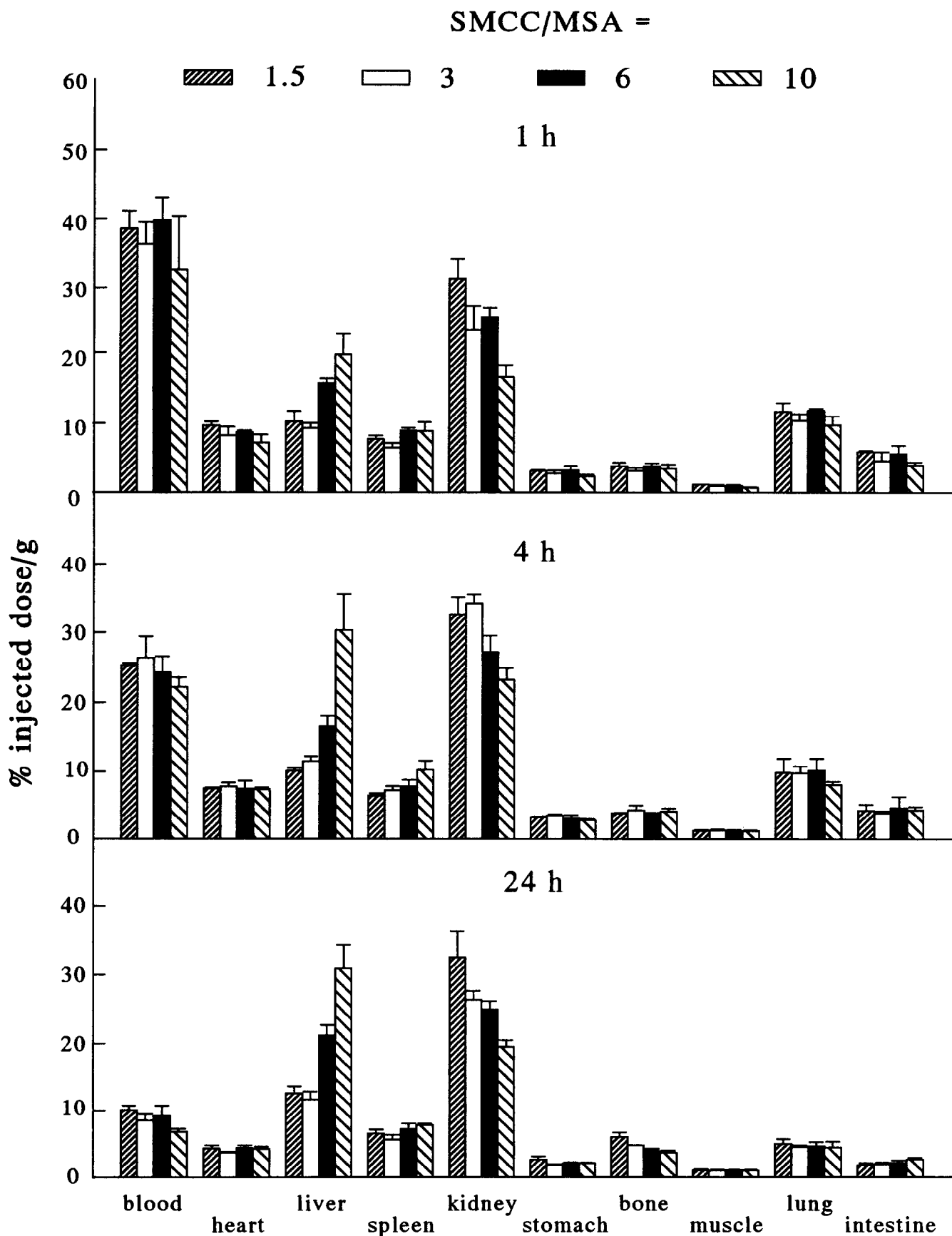
FIG. 6. Biodistribution in BALB/c mice ($n = 3$) of ^{88}Zr-Df-MSA prepared with different SMCC/MSA ratios.

implicates the preference of a small amount of chelating groups in the protein. Conversely, labeling efficiencies are directly related to the amount of chelating groups. Thus, for each protein the golden mean has to be found.

The amount of ^{88}Zr in bone after injection of ^{88}Zr-Df-MSA hardly increased in time and is small when compared with the injection of ^{88}Zr-citrate (Fig. 6). This can be an indication that the Zr-Df-MSA conjugate is relatively stable.

In conclusion, we have shown that it is possible to label proteins with Zr-isotopes. The conjugates have a high in vitro stability and a reasonable good in vivo stability, and they retain their in vivo behavior, as was shown for the model protein MSA.

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