PHYSICS

Deferoxamine, A Promising Bifunctional Chelating Agent for Labeling Proteins with Gallium: Ga-67 DF-HSA: Concise Communication

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A new efficient method has been developed for the labeling of various biologically important proteins with radiogallium, using deferoxamine (DF) as a bifunctional chelating agent. Human serum albumin (HSA) was chosen for studying the DF coupling reaction by a glutaraldehyde two-step method. The DF-HSA conjugate obtained was then easily labeled with Ga-67 with high efficiency and reproducibility. High stability of the Ga-67 DF-HSA was observed both in vitro and in vivo. In rats the half-time of blood clearance at the first phase was 234 min for the Ga-67 DF-HSA, whereas most conventional I-131 HSA gives 121 min. The excellent pharmacological and physiological properties were assessed by plasma clearance and plasma volume measurements in normal volunteers. Even at 2 hr after injection, (90.0 ± 3.0)% of Ga-67 DF-HSA was retained within the vascular space. The mean plasma volume per kilogram of body weight, measured with Ga-67 DF-HSA, was 46.6 ± 3.4 ml/kg, compared with 52.2 ± 2.4 ml/kg as determined with I-131 HSA in the same men.

J Nucl Med 23: 909–914, 1982

In recent years, major emphasis has been placed on procedures for radiolabeling various biologically important proteins with metallic radionuclides using bifunctional chelating agents (*1*,*2*).

Among various potentially applicable agents, we have studied deferoxamine (DF). This is a well-known specific iron-chelating agent of low molecular weight and low toxicity (*3*). It is a commercially available agent currently in use for the treatment of iron overload (*4*,*5*).

Although few studies on Ga-DF chelate have been reported, it appears that Ga^{3+} ion, like Fe^{3+} ion, forms a stable 1:1 chelate (*6*) by coordination through three hydroxamate groups of DF (Fig. 1). The higher affinity of Ga for DF than for the iron-binding macromolecules, lactoferrin and transferrin, has been assessed by Weiner et al. (*7*). In addition, DF forms an uncharged chelate of compact structure, so its influence on the parent protein is minimized.

Moreover, DF contains in its molecule (Fig. 1) an amino group of comparatively high reactivity, regarded as an available functional group for coupling reactions with proteins. Another favorable feature of DF, contrasting with the EDTA or DTPA derivatives, is its low chelate stability with Ca^{2+} or Mg^{2+} cations (*3*). These

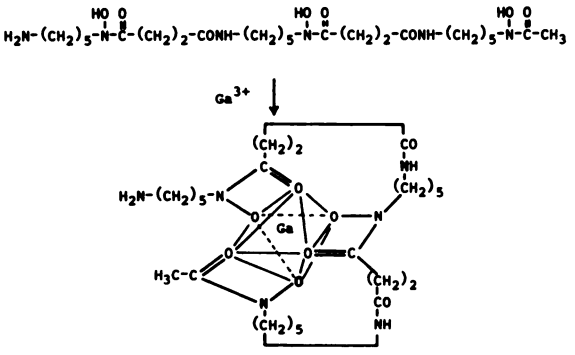

FIG. 1. Structures of deferoxamine (DF) and Ga-DF chelate.

Received Sept. 16, 1981; revision accepted June 10, 1982.

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are essential for many biological reactions, so the low affinity of DF for them is an advantage.

Thus, the great ability of DF to form a stable Ga chelate, and its capability for protein conjugation with minimum influence on the biological properties of the protein, fulfill the characteristics needed for a bifunctional chelating agent. Moreover, it satisfies the general properties required of diagnostic agents, such as low toxicity and easy procurement. These features are regarded as promising for the labeling of various biologically interesting proteins with Ga-67, or with Ga-68, a positron emitter.

For basic studies of DF as plausible bifunctional agent, the preparation of DF-coupled human serum albumin (DF-HSA) is carried out first, followed by its labeling with Ga-67. The in vitro and in vivo behavior are evaluated and compared with the commercially available I-131-labeled HSA (I-131 HSA), followed by the plasma clearance and plasma volume measurements in normal volunteers.

MATERIALS AND METHODS

Coupling of DF to HSA. A glutaraldehyde two-step method (*8*) was modified as follows: A 0.12 *M* solution (1.0 ml) of DF mesylate* was prepared in PBS buffer (0.1 *M* phosphate, 0.14 *M* NaCl, pH 7.4). Next, 0.05 ml of a 20% glutaraldehyde solution was added and stirred for 10 min at room temperature. Then an appropriate volume of this reaction mixture, according to the DF-to-HSA molar ratios needed, was added to 20.0 ml of a 0.2 *mM* solution of HSA† (HBs antigen-free) previously prepared in PBS and kept at 0–4°C. The resultant mixture was stirred for 1 hr at 0–4°C, then, finally, 5.0 mg of sodium borohydride was added and stirred for another 1 hr at 0–4°C.

The DF-HSA preparation was purified by gel chromatography using a Sephadex G-50 column (5.0 × 20 cm), eluted with PBS at a flow rate of 100 ml/hr. The DF-HSA fraction (160–200 ml), separated from free DF (350–450 ml), was kept in a refrigerator.

The concentrations of the protein and DF coupled to HSA were measured by ultraviolet absorbance at 280 nm and by the method of Emery and Hoffer (*9*). Then the number of DF molecules coupled to HSA per molecule was calculated.

Labeling of DF-HSA with Gallium-67. Carrier-free $^{67}GaCl_3$ in 0.01*N* HCl solution (1–5 mCi/ml) of high purity, containing $< 10^{-12}$ *M* Fe^{3+} was used. The required amount of $^{67}GaCl_3$ solution was mixed with 1.0 ml of the DF-HSA solution (10^{-4} *M*). After incubation for 15 min at room temperature, the solution was passed through a 0.22 μm Millipore filter.

Radiochemical purity and in vitro stability. Radiochemical purity of Ga-67-labeled DF-HSA was assayed by gel chromatography using a Sephadex G-50 column, 2.0 × 30 cm, eluted with 0.9% NaCl solution at a flow rate of 80 ml/hr. The eluate was monitored by a flow-through uv detector and a NaI scintillation detector connected to an analog ratemeter system to give a continuous graph of the uv and radioactivity profiles eluted from the column.

In vitro stability of Ga-67 DF-HSA was tested at 37°C. After incubation for 6 and 24 hr, samples were withdrawn and assayed by the above-mentioned gel chromatography system.

Animal studies. Blood clearance studies of Ga-67 DF-HSA were carried out in male Wistar rats weighing 305–330 g, under pentobarbital anesthesia. Ten μCi of Ga-67 DF-HSA were administered intravenously through an exposed femoral vein. Serial blood samples from a catheter inserted in the carotid artery were weighed, and the radioactivity was counted in a NaI well scintillation counter.

The blood radioactivity level at time zero was obtained by extrapolation of the best-fitted line drawn on a semi-logarithmic plot of the first 30 min of data. Blood clearance was then expressed as percentage of the time-zero activity.

For comparison, a similar study was carried out using conventional I-131 HSA‡ (5 μCi).

Biodistribution. Two μCi of Ga-67 DF-HSA was injected intravenously into male ddY mice (25–30 g) through a lateral tail vein. After the desired time interval, each animal was killed and the organs were removed, weighed, and counted in a well counter. Data were expressed as percentage of the injected dose per gram of tissue. For comparison, 2 μCi each of conventional I-131 HSA, Ga-67 DF, and $^{67}GaCl_3$ were also studied.

Effect of storage. The in vitro and in vivo characteristics of Ga-67 DF-HSA were reassessed one and two months after the DF-HSA preparation and storage in a refrigerator.

Preclinical studies in human subjects. Gallium-67 DF-HSA (20 μCi), prepared under sterile conditions, was injected i.v. into six healthy volunteers. Precisely timed heparinized blood samples were taken at 10, 20, 30, 60, and 120 min after injection. One ml of plasma taken from every sample was assayed in a well counter. Data were expressed as a percentage of the time-zero activity level obtained by extrapolation.

Plasma volume of five healthy men was measured with Ga-67 DF-HSA and, two weeks later, with I-131 HSA. One ml of plasma from blood samples obtained at 10 min after injection, as well as 1 ml of diluted standard solution, were counted and used for the plasma volume determination as follows:

$$\text{Plasma volume} = \frac{\text{cpm of 1 ml standard}}{\text{cpm of 1 ml plasma}} \times \text{dilution factor.}$$

TABLE 1. CORRELATION BETWEEN INITIAL DF-TO-HSA MOLAR RATIO IN COUPLING REACTION AND OBTAINED CONJUGATION LEVEL

DF-to-HSA molar ratios		
Coupling condition	Conjugation level Mean	(range, n)
5	0.3	(0.2–0.4, 2)
12	0.8	(0.7–0.9, 6)
15	1.2	(1.0–1.3, 3)
20	1.6	(1.5–1.7, 2)

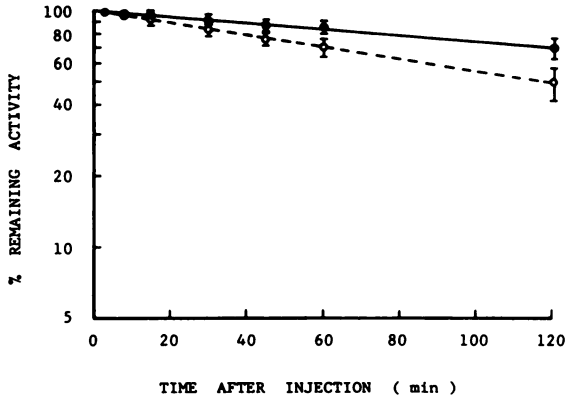

FIG. 3. Blood clearance curves (semilog) for Ga-67 DF-HSA (solid line) and I-131 HSA (dashed) in rats. Mean ± s.d. for 5–6 animals.

RESULTS

Following general and conventional basic considerations for coupling of ligands to albumin, the glutaraldehyde two-step procedure described under Methods was used. The covalent binding of DF to HSA was easily achieved with good efficiency under mild conditions—i.e., at neutral pH, in aqueous medium, and at a temperature of 0–4°C—within a fairly short time. The correlation between the initial amount of DF in the reaction medium and that of DF actually coupled to HSA is summarized in Table 1 in terms of DF-to-HSA molar ratios. The conjugation level of an average of 0.8 mole of DF per mole of HSA, achieved from the initial 12 times molar excess of DF, was chosen for the present study.

The labeling was easily performed by simple incubation, at pH 7–8, of the conjugate solution with highly purified carrier-free $^{67}GaCl_3$ solution. Radiochemical purity of this Ga-67-labeled DF-HSA was assayed by the Sephadex G-50 gel chromatography. A typical elution profile is shown in Fig. 2A. A single peak corresponding to the Ga-67 DF-HSA (17–27 min) was detected. No free Ga-67 DF-HSA chelate (43–57 min) was detected. This indicated that the labeling reaction proceeded quantitatively. A labeling yield of 99.8 ± 0.3% was obtained in the range of Ga-67 activity tested (0.1–5.0 mCi), with a specific radioactivity of 0.015–0.75 mCi/mg of albumin.

In vitro stability of the Ga-67 DF-HSA was assayed by incubation of the preparation at 37°C for 6 or 24 hr. After 24 hr, a negligibly small peak (50 min) could be detected (Fig. 2C) on the gel chromatogram.

In vivo stability was determined by comparative studies of blood clearance of the Ga-67 DF-HSA and conventional I-131 HSA in rats, as shown in Fig. 3. The Ga-67 DF-HSA showed higher blood levels for a longer period of time than I-131 HSA. The clearance half-time of the first phase was 234 min for Ga-67 DF-HSA, and 121 min for I-131 HSA. A similar phenomenon was observed in biodistribution studies in mice. The blood disappearance of Ga-67 DF-HSA was slower than that of I-131 HSA, even at 3 hr after injection (Tables 2 and 3). The I-131 radioactivity in the stomach increased with time, indicating the presence of deiodination (Table 3). The results of biodistribution studies of Ga-67 DF and $^{67}GaCl_3$ in mice are summarized in Tables 4 and 5, respectively.

The remarkable stability of Ga-67 DF-HSA was reassessed by plasma clearance in healthy subjects. The mean remaining activity of Ga-67 DF-HSA was (90.0 ± 3.0)% even 2 hr after injection, and average plasma volume per kilogram of body weight measured was 46.6 ± 3.4 ml/kg, as opposed to the 52.2 ± 2.4 ml/kg obtained with I-131 HSA, leading to a mean percentage difference in plasma volume between the two of −10.9 ± 2.7% with I-131 HSA taken as the reference.

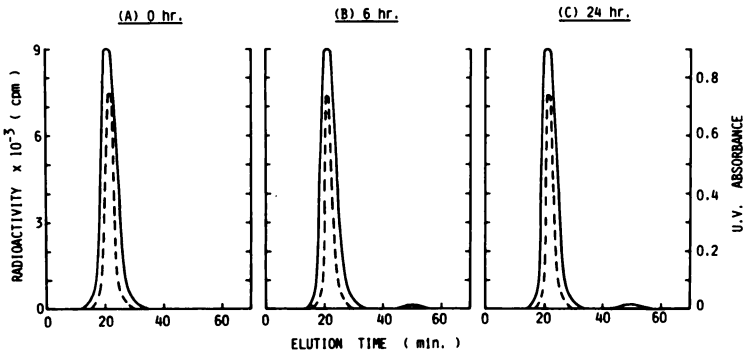

FIG. 2. Sephadex G-50 gel chromatograms of Ga-67 DF-HSA obtained: (A) immediately after labeling, and (B) at 6 hr, and (C) 24 hr after incubation at 37°C. Solid line = radioactivity profile; dashed = uv absorbance.

TABLE 2. BIODISTRIBUTION OF RADIOACTIVITY IN MICE AFTER INTRAVENOUS INJECTION OF Ga-67 DF-HSA

	% Injected dose per gram tissue*			
	30 min	1 hr	3 hr	6 hr
Blood	32.40 ± 2.48	30.62 ± 2.49	25.15 ± 1.75	15.77 ± 1.51
Liver	6.99 ± 0.53	8.61 ± 0.48	7.99 ± 0.49	7.53 ± 1.56
Kidney	8.70 ± 0.73	9.65 ± 0.97	9.49 ± 0.84	7.84 ± 0.99
Intestine	1.42 ± 0.45	2.79 ± 0.44	3.34 ± 0.41	3.09 ± 1.56
Stomach	1.14 ± 0.09	0.80 ± 0.02	1.06 ± 0.34	1.08 ± 0.50
Spleen	3.95 ± 0.81	5.61 ± 0.86	5.32 ± 0.52	4.45 ± 0.87
Lung	10.02 ± 1.13	9.22 ± 0.44	11.12 ± 0.78	9.76 ± 2.69

* Mean ± 1 s.d. for 5 animals each point.

TABLE 3. BIODISTRIBUTION OF RADIOACTIVITY IN MICE AFTER INTRAVENOUS INJECTION OF I-131 HSA

	% Injected dose per gram tissue*			
	30 min	1 hr	3 hr	6 hr
Blood	32.85 ± 4.25	27.36 ± 4.37	21.36 ± 4.77	15.61 ± 1.05
Liver	7.96 ± 2.99	7.81 ± 1.21	3.83 ± 0.72	3.87 ± 1.13
Kidney	9.56 ± 1.09	7.57 ± 0.60	5.77 ± 0.36	4.92 ± 0.29
Intestine	1.73 ± 0.07	1.89 ± 0.16	1.96 ± 0.22	1.83 ± 0.41
Stomach	3.35 ± 0.84	3.99 ± 1.11	7.45 ± 1.35	10.03 ± 4.77
Spleen	4.80 ± 0.79	3.84 ± 0.90	2.85 ± 0.38	2.71 ± 0.46
Lung	10.63 ± 2.42	8.60 ± 1.41	7.41 ± 0.93	7.22 ± 1.23

* Mean ± 1 s.d. for 5 animals each point.

TABLE 4. BIODISTRIBUTION OF RADIOACTIVITY IN MICE AFTER INTRAVENOUS INJECTION OF Ga-67 DF

	% Injected dose per gram tissue*			
	30 min	1 hr	3 hr	6 hr
Blood	0.38 ± 0.15	0.10 ± 0.01	0.06 ± 0.02	0.05 ± 0.01
Liver	0.27 ± 0.07	0.17 ± 0.01	0.26 ± 0.15	0.17 ± 0.01
Kidney	3.60 ± 0.33	2.66 ± 0.26	2.73 ± 0.60	2.13 ± 0.27
Intestine	0.25 ± 0.09	0.16 ± 0.02	0.22 ± 0.05	0.36 ± 0.25
Stomach	0.06 ± 0.03	0.07 ± 0.05	0.11 ± 0.11	0.03 ± 0.02
Spleen	0.22 ± 0.19	0.07 ± 0.04	0.07 ± 0.06	0.02 ± 0.02
Lung	0.41 ± 0.14	0.12 ± 0.02	0.09 ± 0.04	0.06 ± 0.01

* Mean ± 1 s.d. for 5 animals each point.

The stability of the DF-HSA conjugates was tested at one and two months after its preparation. Each labeled conjugate showed in vitro and in vivo characteristics like those in the fresh preparation.

DISCUSSION

The mild coupling condition and the easy and rapid labeling reaction indicate that the selection of DF as a bifunctional chelating agent will provide a useful means of labeling biologically active molecules.

By the stepwise coupling procedure selected—that is, the reaction of DF with one carbonyl group of the glutaraldehyde first, followed by the attachment of the HSA through the other carbonyl group—the often-mentioned protein polymerization (*10*) was avoided. Moreover, no reductive cleavage of disulfide protein bonds was detected, due to the low concentration of sodium borohydride used (*11*).

TABLE 5. BIODISTRIBUTION OF RADIOACTIVITY IN MICE AFTER INTRAVENOUS INJECTION OF $^{67}GaCl_3$

	% Injected dose per gram tissue*			
	30 min	1 hr	3 hr	6 hr
Blood	18.82 ± 4.39	13.45 ± 2.35	10.62 ± 4.46	3.71 ± 0.83
Liver	4.15 ± 0.85	3.49 ± 0.49	4.14 ± 1.49	2.84 ± 0.52
Kidney	5.81 ± 1.19	4.71 ± 0.86	5.03 ± 2.66	2.59 ± 0.43
Intestine	2.41 ± 0.55	2.55 ± 0.59	5.25 ± 1.50	4.24 ± 0.73
Stomach	2.07 ± 0.49	1.79 ± 0.10	1.55 ± 0.20	1.66 ± 0.40
Sleen	3.99 ± 0.87	3.46 ± 0.77	4.58 ± 0.90	2.22 ± 0.41
Lung	7.52 ± 2.33	8.49 ± 2.06	6.73 ± 4.32	2.64 ± 0.79

* Mean ± 1 s.d. for 5 animals each point.

It is known that the level of conjugation, the molar ratio of coupled chelating group to protein, affects both the biological properties and the specific radioactivity of the labeled protein. Thus, since it is desirable to minimize the level of conjugation to preserve biological integrity, but to maximize it in terms of specific radioactivity, the study was carried out at a conjugation level of 0.7–0.9, the optimum range reported for HSA (*12,13*).

Labeling of DF-HSA with Ga-67 was an easy procedure accomplished by simple mixing of $^{67}GaCl_3$ with the DF-HSA conjugate at room temperature. Since a very high labeling yield was obtained, further purification, frequent step in radioiodination, was unnecessary. A high labeling efficiency, near 99.8%, with a specific activity of 0.015–0.75 mCi/mg, was obtained at neutral pH.

The high in vitro as well as in vivo stability of Ga-67 DF-HSA is well illustrated by the results presented in Figs. 2, 3, 4, and Table 2. Also, the results of mouse biodistribution studies (Table 2, 4, and 5), as well as the human experiments (Fig. 4 and Table 6) clearly show that Ga-67 DF-HSA behaves as an entity in the living body, with no cleavage of Ga-67 DF chelates: no Ga-67, either free or bound to transferrin, was seen over the period of study.

Moreover, the comparisons with I-131 HSA showed that the deteriorating effect of conjugation and labeling on the biological properties of HSA was less than that caused by the conventional radioiodination (Figs. 3 and 4, Table 2); thus the physiological and pharmacological properties of the protein molecule are preserved.

In conclusion, the design of a DF based radiopharmaceutical has shown excellent capability for the labeling of albumin with radiogallium. The usefulness of this nontoxic DF as a bifunctional chelating agent is remarkable, since it needs only a very easy and proficient coupling reaction with a mild glutaraldehyde. With the production of a very stable DF-HSA conjugate, the labeling reaction was easily performed leading to a Ga-67 DF-HSA of excellent in vitro and in vivo characteristics.

This work with albumin provided a basis for understanding and planning its application to the labeling of more important biological molecules such as fibrinogen, plasminogen, or antibodies. Studies with Ga-67-labeled DF-coupled fibrinogen have already shown excellent performances for the imaging of deep-vein thrombi, in-

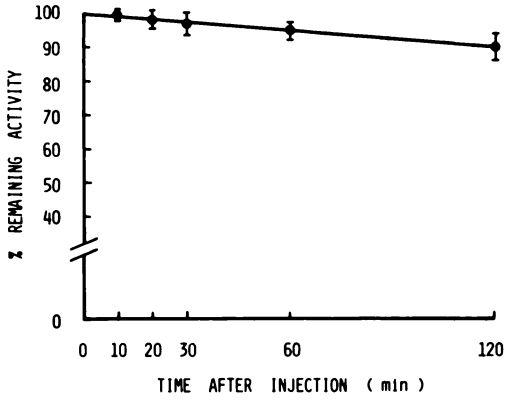

FIG. 4. Ga-67 DF-HSA clearance (linear) from plasma in six human subjects (mean ± s.d.).

TABLE 6. PLASMA VOLUME MEASUREMENTS WITH Ga-67 DF-HSA AND I-131 HSA

Volunteer No.	Ga-67 DF-HSA (ml/kg)	I-131 HSA (ml/kg)	Difference* (%)
1	44.4	50.9	−12.8
2	43.5	50.2	−13.6
3	46.7	52.9	−11.7
4	52.3	56.2	−6.9
5	46.2	51.0	−9.4
Mean ± 1 s.d.	46.6 ± 3.4	52.2 ± 2.4	−10.9 ± 2.7

* (Ga-67 DF-HSA—I-131 HSA) × 100/I-131 HSA.

dicating promise for future applications of this new bifunctional chelating agent (*14*).

FOOTNOTES

* Desferal, CIBA Pharmaceutical Co., Summit, NJ.
† Alpha Therapeutic Co., South Pasadena, CA.
‡ Commissariat A L'Energie Atomique, Sorin 13040, Saluggia.

ACKNOWLEDGMENT

This work was supported in part by a grant from the Ministry of Health and Welfare, Japan.

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