Investigation of Physicochemical and In-Vivo Behavior of Diastereomeric Iron-59, Gallium-68, and Indium-111-EHPG Trivalent Metal Complexes

Susan L. Madsen, Christopher J. Bannochie, Arthur E. Martell, Carla J. Mathias, and Michael J. Welch

Edward Mallinckrodt Institute of Radiology, Washington University Medical School, St. Louis, Missouri and Department of Chemistry, Texas A&M University, College Station, Texas

EHPG (N, N'-ethylenebis-[2-(*o*-hydroxyphenyl)glycine]) trivalent metal complexes show diagnostic potential both in radiopharmaceutical applications (gallium-68, indium-111) and as MRI contrast agents (iron). Although several publications report similar behavior of iron, gallium and indium complexes, we observe significant differences between EHPG complexes of these metals both in physicochemical and in vivo analyses. In addition, stereospecific behavior has been observed for the two diastereomers of EHPG when complexed with each metal. Complexes of Fe[^{59}Fe], ^{68}Ga, and ^{111}In with meso, racemic, and unseparated EHPG have been evaluated. Relative lipophilicity has been measured with HPLC. Blood clearance and liver uptake have been correlated with pM values, stability constants and metal ion reduction potentials.

J Nucl Med 1990; 31:000–000

The hexachelating ligand EHPG (N,N'-ethylenebis-[2-(*o*-hydroxy-phenyl)glycine]) has been suggested as a ligand with applications for use as an MRI contrast agent when complexed with trivalent iron (*1*). Trivalent metal complexes of this strong chelate may also be useful for both gallium-68 (^{68}Ga) positron emission tomography (PET) and indium-111 (^{111}In) single-photon emission computed tomography (SPECT). Each of these radio-metal complexes has been evaluated. For purposes of comparison, we have also investigated ^{59}Fe-EHPG.

Commercially available EHPG has been shown to contain a mixture of approximately 1:1 meso:racemic diastereomers of this ligand (*2*). The racemic diastereomer is an unresolved mixture of [R,R] and [S,S] enantiomers. We refer to unseparated EHPG when discussing a sample in which the meso and racemic diastereomers have not been separated from one another. The large majority of existing EHPG literature considers only unseparated EHPG (*3–10*). By not addressing the issue of stereoisomerism, these determinations report results of a system which contains a mixture of different compounds. We have evaluated the physicochemical and in vivo behavior of each diastereomer and compared the results with that of unseparated EHPG in order to note any significant differences.

Received Oct. 30, 1989; revision accepted Apr. 10, 1990.

For reprints contact: Michael J. Welch, Mallinckrodt Institute of Radiology, Washington University Medical School, Division of Radiation Science, Box 8131, 510 S. Kingshighway, St. Louis, MO 63110.

MATERIALS AND METHODS

The gallium (111) chloride and indium (111) chloride used in carrier-added experiments were obtained from Aldrich Chemical Company (Milwaukee, WI). HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid) was obtained from Research Organics, Inc. (Cleveland, OH). DTPA (Diethylenetriaminepentaacetic acid) was obtained from Sigma Chemical Company (St. Louis, MO). Kieselgel silica-60 thin-layer chromatography glass plates were obtained from EM Sciences. All other chemicals and solvents were obtained from Fisher Scientific (Fair Lawn, NJ). Chemicals and solvents were used without further purification.

Radioactive Materials

Iron-59 ferric chloride was obtained in 0.5-*M* HCl (E.I. DuPont de Nemours & Co., Inc., N. Billerica, MA) with a specific activity generally in the range of 30–40 mCi/mg. No-carrier-added ^{68}Ga-chloride was obtained in 1-N HCL from a 100 MCi ^{68}Ge/^{68}Ga generator (E. I. DuPont de Nemours & Co., Inc.). No-carrier-added ^{111}In-chloride was obtained in aqueous sodium chloride, pH = 1-3 (Medi-Physics, Inc., Richmond, CA and Mallinckrodt Medical, Inc., St. Louis, MO).

Preparation of Radioactive Complexes

The ligands which were evaluated are meso-EHPG, racemic-EHPG and unseparated-EHPG. The method of isolation of each diastereomer has been described in detail in previous works (*11*). Unseparated-EHPG was obtained from Dojindo Labs (Japan) under the alternate name ethylenediamine-N,N'-bis[α-(2-hydroxyphenylacetic acid)] (EDDHA). Radioactive complexes of M (M = Fe[^{59}Fe], ^{68}Ga or ^{111}In)

were prepared with each ligand as described below. A 1:1 M-meso-EHPG:M-racemic-EHPG mixture was prepared by combining equal amounts of radioactivity (regardless of volume) of separate solutions of M-meso-EHPG and M-racemic-EHPG.

All of the radioactive metal complexes were prepared by the following method. The desired amount of radioactivity (metal chloride solution) was evaporated to dryness under N_2 with a 700°C heat gun. For carrier-added solutions, the desired amount of carrier metal chloride solution ($\sim 4.0 \times 10^{-9}$ moles of M) was added to the radioactive metal chloride solution before evaporation. In cases where the total volume of metal chloride solution was <10 μl, no evaporation was performed. One hundred to 200 μl 0.4-*M* sodium acetate (pH = 5.75) was added directly to the evaporation residue to resolubilize the radioactive metal as the acetate complex (pH = 5-6).

A 1-m*M* solution of the desired ligand was prepared in 0.05-*M* sodium hydroxide. EHPG chelation to the metal was achieved by adding 300–600 μl of the ligand solution to the metal acetate complex in solution. Generally, a volume/volume ratio of 3:1 ligand:metal acetate complex was used. The pH was then adjusted to 7–8 with 1-N HCl.

An ^{111}In-DTPA standard was prepared for electrophoresis. The ^{111}In-acetate complex was prepared in solution as described above. To this was added a 1-m*M* solution of DTPA in the same volume ratios as the EHPG complexes. The pH of the mixture was adjusted to 7–8. The solution was allowed to stand 10 min at ambient temperatures before running on electrophoresis.

Analysis of Complexes

The exchange reaction between EHPG and metal acetate was monitored with electrophoresis (*12*). Samples (3 μl) were run simultaneously with an ^{111}In-DTPA standard on cellulose acetate strips (Gelman Sciences). Metal acetate complexes and metal trihydroxides have no charge and no migration is observed. At pH = 7.35, the EHPG complexes have a negative charge and migrate approximately 35% of the distance travelled by ^{111}In-DTPA (a dianion) in the same system. In this way, 100% radiochemical yields were observed (all of the radioactive metal complexed with EHPG). We see no evidence of metal tetrahydroxides.

High-performance liquid chromatography (HPLC) analysis was used to characterize meso- and racemic-EHPG metal complexes. A styrogel (PRP-1, Hamilton Company, Reno, NV) reverse-phase column (4.1 mm × 150 mm) coupled with a Spectraphysics (SP8700) solvent delivery system was used. The samples were eluted with 50:50 0.05-*M* sodium citrate/methanol (HPLC grade), pH = 7.10, at a flow rate of 1.00 ml/min (*13*). Mass was detected at 295 nm with a variable wavelength UV detector (GM770R, Kratos Analytical Instruments, Arlington Heights, IL). Radioactivity was detected with a NaI(Tl) crystal and monitored with a time-mode multichannel analyzer (Series 35 Plus, Canberra, Meriden, CT).

Animal Studies

Doses for animal studies were prepared by diluting the radioactive complex solution to the desired volume with 0.9% sodium chloride (saline). Generally, 0.1 ml per animal plus one standard dose was prepared. Doses of ~3, 5, or 50 μCi for Fe[^{59}Fe]-EHPG, ^{111}In-EHPG, and ^{68}Ga-EHPG, respectively, were injected.

The biodistribution studies were performed in mature female Sprague-Dawley rats (150–200 g). Animals were allowed free access to food and water ad libitum. The radioactive complex was injected in a surgically exposed femoral vein of an anesthetized (ether inhalation) rat. Unseparated-EHPG and mixed samples were injected within a maximum time frame of 30 min post-complexation. The wound was clipped to close and the animal was allowed to recover. At the appropriate time postinjection, the animal was reanesthetized with ether and killed by decapitation. The organs of interest were removed and weighed. The radioactivity in organ samples was determined using an automatic well-type gamma counter (Beckman, Gamma 8000). The standard dose was diluted, weighed, and counted. The amount of radioactivity injected was calculated by comparing the injected dose with the standard dose by weight.

RESULTS

Each radioactive metal was obtained as a trichloride complex in acid medium. EHPG is soluble in aqueous medium only under basic conditions. In order to avoid formation of the highly insoluble metal hydroxides, all metal chlorides were converted to metal acetate before EHPG complexation was attempted. Since the acetate complexes are soluble in a basic aqueous environment, an exchange reaction from metal acetate to EHPG complex can be achieved quickly and easily at room temperature under slightly basic conditions (pH = 7–8).

HPLC experiments demonstrate significant differences in physical characteristics of the two diastereomers and the metal complexes. HPLC retention times (Table 1) indicate a change in complex interactions when one trivalent metal is substituted for another in a metal-ligand complex. Samples of unseparated EHPG complexes show HPLC retention of both meso-EHPG and racemic-EHPG complexes. The ratio of meso- to racemic-EHPG complex can be measured by integration of the amount of radioactivity in each peak. To allow for better separation of the radioactivity peaks in unseparated-EHPG metal complex solutions, two similar HPLC columns were set up in sequence in order to increase the time between detection of each diastereomeric complex within the sample. Figure 1 shows that at ambient temperatures Fe[^{59}Fe]-unseparated-EHPG

TABLE 1
HPLC Elution Times (min)

Free ligand	R_t (x ± s.d.; n = 5)	
	Meso*	Racemic*
	6.37 ± 0.01	4.51 ± 0.01
Fe	6.18 ± 0.01	4.51 ± 0.02
Ga	5.21 ± 0.01	4.39 ± 0.01
In	5.81 ± 0.33	5.27 ± 0.01

* Based on UV mass detection.

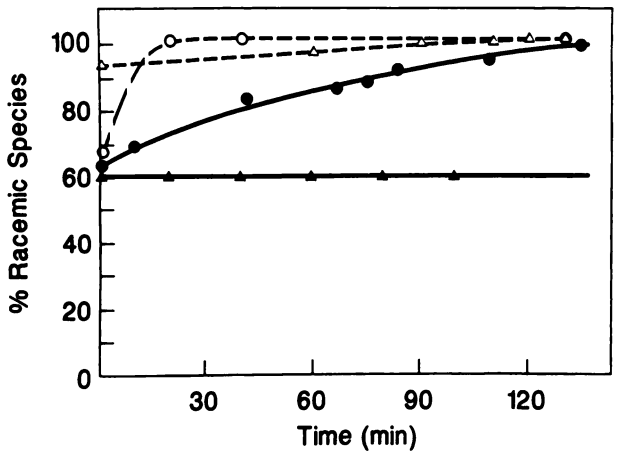

FIGURE 1
Rate of M-racemic-EHPG formation from excess unseparated-EHPG; (●) M = Fe[^{59}Fe]; (▲) M = ^{68}Ga at 25°C; (○) M = Fe[^{59}Fe]; and (△) M = ^{68}Ga at 82°C. % Species was calculated from integration of radioactivity peaks generated by HPLC.

in excess ligand contains 63% racemic-EHPG complex 1 min post-addition of ligand to metal acetate. After 2 hr at room temperature (occasional mixing), all of the ^{59}Fe is complexed with the racemic diastereomer.

Gallium and indium have 61% and 66%, respectively, of the radioactive metal complexed with the racemic diastereomer one minute post addition of ligand at ambient temperature. This percentage is only of the radioactivity which has exchanged to EHPG at that time. For these metals, no exchange to the racemic complex was evident within 2 hr post-ligand addition at room temperature. Figure 1 shows a more rapid equilibration for iron complexes at 82°. The higher temperature allows equilibration of the gallium solution to 100% ^{68}Ga-racemic-EHPG within 2 hr. However, there is still no significant exchange to the racemic metal complex for ^{111}In-unseparated-EHPG.

The biodistribution studies (Tables 2–4) show that for each metal, the meso-EHPG complex clears the blood more slowly than the racemic-EHPG complex. With iron and gallium, there is higher liver uptake for meso-EHPG complex than for racemic-EHPG complex while the reverse is true for indium complexes.

More rapid blood clearance is observed for gallium complexes relative to iron and indium. When compared with iron, gallium and indium complexes show a larger percentage of the radioactivity which enters clearance organs clearing through the kidney rather than through the liver. The rate of uptake and clearance of meso-EHPG complexes in the liver was studied out to 2 hr postinjection (Fig. 2). Fe[^{59}Fe]-meso-EHPG and ^{111}In-meso-EHPG complexes show only slight clearance of radioactivity from the liver over this time period. Gallium-68 meso-EHPG complexes clear rapidly. For all of the metals, it appears that as the radioactivity leaves the liver it clears through the intestinal tract.

Carrier-added solutions of ^{68}Ga and ^{111}In complexes were prepared in order to validate the in vivo comparison of carrier-added Fe[^{59}Fe]-EHPG with the carrier-free isotopes. Carrier-added Ga[^{68}Ga]-EHPG complexes behaved the same in vivo as did the carrier-free ^{68}Ga-EHPG ($p > 0.10$). Carrier-added In[^{111}In]-EHPG biodistribution data agree within experimental error with carrier-free ^{111}In-EHPG ($p > 0.50$) except liver and kidney ($0.02 < p < 0.05$). In [^{111}In]-meso-EHPG shows higher uptake in the liver at 1 hr postinjection than carrier-free ^{111}In-meso-EHPG while the kidney %ID/g data agree within experimental error. Several repeat In[^{111}In]-EHPG carrier-added experiments were carried out. In experiments where kidney %ID/g agreed with carrier-free ^{111}In-EHPG, the liver radioactivity values did not. However, frequently, carrier complex experiments gave results where liver values did agree within experimental error with carrier-free liver data, but the kidney radioactivity values did not. Due to the number of animals per study, carrier-free and carrier-added studies were not paired. Minor differences in metabolism and/or complex to transferrin metal exchange rate could account for the small, albeit statistical, differences obtained. At the concentration levels of the Fe[^{59}Fe]-EHPG complexes, the carrier-added studies show no change in behavior of ^{68}Ga-EHPG, and only the minor changes in ^{111}In-EHPG discussed above. Thus, our comparisons of carrier iron with carrier-free gallium and indium complexes are valid.

DISCUSSION

The ratio of the diastereomers in unseparated EHPG ligand has been shown to be roughly 1:1 (*2*). In the presence of excess unseparated-EHPG relative to metal, more racemic-EHPG complex is formed (Fig. 1). This

TABLE 2
Iron-59-EHPG Biodistribution %ID/g 1 Hour Postinjection (x ± s.d.; n = 5)

Organ	Meso	Racemic	Unseparated	1:1 mix
Blood	2.034 ± 0.116	0.677 ± 0.137	1.043 ± 0.191	1.354 ± 0.143
Liver	1.219 ± 0.191	0.495 ± 0.174	0.900 ± 0.252	1.007 ± 0.356
Spleen	0.521 ± 0.115	0.203 ± 0.138	0.330 ± 0.070	0.502 ± 0.274
Kidney	1.627 ± 0.150	0.663 ± 0.240	0.998 ± 0.315	1.863 ± 0.325
Muscle	0.252 ± 0.015	0.225 ± 0.091	0.193 ± 0.029	0.252 ± 0.073

TABLE 3
Gallium-68-EHPG Biodistribution %ID/g 1 Hour Postinjection (x ± s.d.; n = 5)

Organ	Meso	Racemic	Unseparated	1:1 mix
Blood	0.071 ± 0.017	0.062 ± 0.019	0.044 ± 0.015	0.071 ± 0.025
Liver	0.404 ± 0.047	0.190 ± 0.060	0.168 ± 0.022	0.266 ± 0.089
Spleen	0.028 ± 0.004	0.027 ± 0.005	0.023 ± 0.003	0.036 ± 0.011
Kidney	1.082 ± 0.353	1.731 ± 0.357	1.538 ± 0.342	2.209 ± 0.811
Muscle	0.029 ± 0.010	0.030 ± 0.015	0.032 ± 0.016	0.058 ± 0.028

could be the result of faster ligand exchange kinetics for the racemic isomer. However, no difference in the rate of complexation of the two diastereomers has been detected by radio-thin layer chromatography (10:2:1 n-BuOH:H_2O:HOAc; Silica-60) of the exchange reaction of ligand for acetate. Another explanation could be the thermodynamic influence of a more stable racemic diastereomeric metal complex.

The complexes are prepared in the presence of excess ligand (at least 10:1 ligand to metal for Fe[^{59}Fe] complexes and approximately 10^8:1 ligand:metal for carrier-free ^{68}Ga and ^{111}In complexes). If equal amounts of metal and unseparated-EHPG were combined in solution, approximately a 1:1 ratio of racemic-EHPG:meso-EHPG metal complexes would form. When an excess of ligand greater than 2:1 ligand:metal is present, it is possible for all of the metal ions to complex exclusively with the more stable diastereomer. Figure 1 indicates that one minute after addition of excess unseparated-EHPG to the metal acetate complex in solution, more of the radioactive metal is bound to racemic-EHPG.

After 1 min, excess racemic-EHPG free ligand continues to compete with the Fe[^{59}Fe]-meso-EHPG complex for the iron. Fe[^{59}Fe]-racemic-EHPG is two orders of magnitude more stable than Fe[^{59}Fe]-meso-EHPG (Table 5). Since the racemic-EHPG metal complex is thermodynamically favored over the meso-EHPG metal complex, the racemic diastereomer competes successfully for the metal. After 2 hr at ambient temperatures, all of the iron has exchanged to racemic-EHPG.

The stabilities of gallium and indium diastereomeric complexes differ by one order of magnitude. This is a significantly smaller difference than in the case of iron. At ambient temperatures, exchange of these metals to the thermodynamically favored (Table 5) racemic-EHPG metal complexes is not evident. The rate of the exchange reaction can be increased by warming the solution to 82°C (Fig. 1). After 30 min at this temperature, all of the iron is exchanged to the racemic diastereomer. Gallium metal is exchanged completely to racemic-EHPG within 2 hr. No significant change in species distribution is evident for the indium solution over 2 hr at 82°C. This indicates that racemic-EHPG competes more successfully against ^{68}Ga-meso-EHPG than against ^{111}In-meso-EHPG. One would predict a larger difference between the stabilities of the gallium diastereomeric complexes than between the indium diastereomeric complexes.

The data in Figure 1 can be used to define the species distribution of the radioactive unseparated-EHPG metal complexes at the time of injection. If an Fe-unseparated-EHPG sample was not injected until 30 min post-completion of the acetate/EHPG exchange, ~80% Fe[^{59}Fe]-unseparated-EHPG would be in the form of Fe[^{59}Fe]-racemic-EHPG. For gallium and indium, the amount of racemic complex present in the unseparated reaction at the time of injection would be between 60%–65%. For this reason, the in vivo behavior of unseparated-EHPG metal complexes is more like that of racemic-EHPG metal complexes than meso-EHPG metal complexes. The in vivo behavior of the 1:1 diastereomeric complex mixture is a better average of the behavior of the two separate diastereomeric metal complexes.

Because of the high affinity of transferrin for metal ions (*14*), the ability of a complex to survive a physiologic environment has sometimes been predicted based on the complex stability constant relative to that of transferrin (*15*). However, since transferrin takes up free metal ions in the blood stream, the behavior of a complex in vivo may be more accurately explained by considering pM values, which, defined by Equation 1, is a direct measure of the amount of free metal ion present at equilibrium.

TABLE 4
Indium-111-EHPG Biodistribution %ID/g 1 Hour Postinjection (x ± s.d.; n = 5)

Organ	Meso	Racemic	Unseparated	1:1 mix
Blood	1.475 ± 0.608	0.623 ± 0.261	0.918 ± 0.355	1.231 ± 0.391
Liver	1.023 ± 0.142	2.417 ± 0.368	1.974 ± 0.273	1.512 ± 0.129
Spleen	0.269 ± 0.120	0.135 ± 0.047	0.175 ± 0.052	0.212 ± 0.072
Kidney	4.504 ± 0.483	14.298 ± 1.831	10.475 ± 0.652	8.595 ± 0.501
Muscle	0.292 ± 0.052	0.173 ± 0.039	0.185 ± 0.026	0.220 ± 0.029

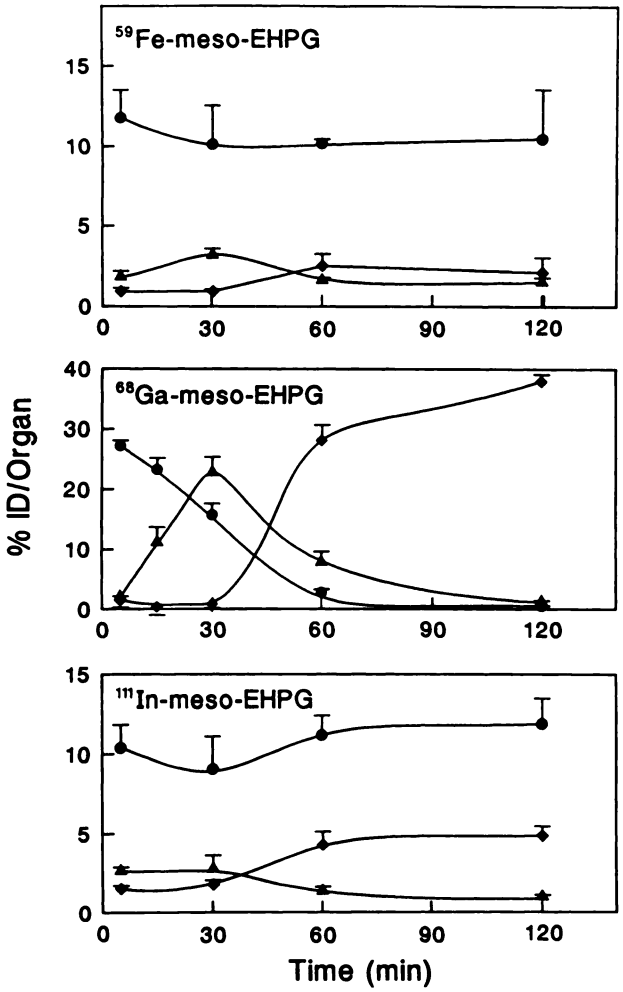

FIGURE 2
Hepatobiliary clearance of M-meso-EHPG out to 2 hr postinjection. Note that while the ^{68}Ga complex clears rapidly from the liver, the ^{59}Fe and ^{111}In complexes do not.

$$M^{+3} + L^{-n} = ML^{3-n}$$

$$pM = -\log[M^{+3}] \quad (1)$$

Unlike stability constants, pM values are conditional. The values listed in Table 5 (Bannochie, Martell, 1989) were calculated for 2:1 ligand:metal ($[M^{+3}] = 1.0 \times 10^{-5}$M) at pH = 7.40. pM values can be calculated from the measured stability constant and the protonation constants of the ligand (*16*). Due to the dependence of pM on ligand protonation constants, very different predictions about in vivo stability can result depending upon whether log K or pM values are compared.

In the case of indium, based upon stability constants, one would expect the complex to be stable in vivo since the In-EHPG stability constants are five to six orders of magnitude larger than transferrin stability constants. However, based upon pM, one would predict a high degree of exchange of the indium metal ion away from the complex to transferrin since both pM values are lower than for In-transferrin. Such a comparison illustrates that different conclusions can be drawn from data depending upon whether log K or pM values are compared. This difference emphasizes the importance of calculating pM values for metal complexes. A more accurate explanation of in vivo behavior can be achieved if one uses pM values to understand in vivo activity rather than stability constants.

TABLE 5
pM Values and Stability Constants*

	meso-EHPG		racemic-EHPG		Transferrin	
Ligand	pM	log K	pM	log K	pM	log K
Fe(III)	23.9	33.28	26.0	35.54	20.7	20.7,19.4†
Ga(III)	23.0	32.40	24.3	33.89	20.4	20.3,19.3‡
In(III)	15.9	25.26	17.1	26.68	18.9	19.2,18.1§

* Bannochie, Martell, 1989.
† $[HCO_3^-] = 1.4 \times 10^{-4}$ M (*22*).
‡ $[HCO_3^-] = 5.0 \times 10^{-3}$ M (*14*).
§ $[HCO_3^-] = 1.4 \times 10^{-4}$ M, as estimated in Ref 11.

For the cases of iron and gallium, either pM or log K would predict a stable EHPG metal complex relative to transferrin metal complex. However, stability constants predict a very large difference in these stabilities (12 log units) while pM values differ by a much smaller margin (3–6 log units).

One can use pM values to compare the two diastereomeric complexes of any metal. For all of the metals studied, meso-EHPG complexes have lower pM values than the corresponding racemic-EHPG complexes. Thus, meso complexes have more free metal ion present in vivo. These free metal ions can bind to transferrin in the blood. The biologic half-lives of M-transferrin complexes in the bloodstream have been measured (*17*) and are significantly longer than those of the EHPG complexes. Thus, the radioactive metals bound to transferrin give the appearance of slower blood clearance. This may explain the greater amount of radioactivity in the blood for meso complexes relative to racemic complexes.

Gallium-68-EHPG exhibits rapid blood clearance relative to indium (Fig. 3). This can be explained based on relative pM values. Indium-111-EHPG has a lower pM than ^{68}Ga-EHPG. Thus, the ^{111}In-EHPG complexes surrender more free metal ions to the bloodstream for transferrin chelation than do the gallium complexes. As a result, the level of radioactivity present in the bloodstream is elevated more by the transferrin interaction for indium than for gallium. Based on pM, one would expect iron complexes to contribute the least number of free metal ions in vivo and, thus, exhibit the most rapid blood clearance rate of all three metals. Instead, iron shows a significant amount of radioactivity in the blood at one hour. One possible explanation for this is that the large amount of iron metal present naturally

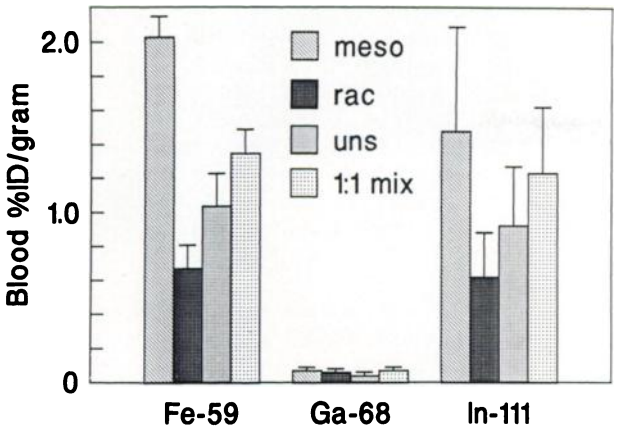

FIGURE 3
M-EHPG blood radioactivity levels at 1 hr postinjection. Note that ^{68}Ga complexes clear the blood much more rapidly than either ^{59}Fe or ^{111}In complexes.

in vivo somehow blocks the relevant mechanism, resulting in a slower clearance rate. A more likely explanation is related to liver activity.

In the liver, ferrous (Fe^{+2}) ions are incorporated into ferritin iron cores by the reaction:

$$4Fe^{+2} + O_2 + 6H_2O \leftrightarrow 4FeOOH + 8H^+ \ (18),$$

where the FeOOH complex contains an oxidized ferric (Fe^{+3}) cation. It has been shown that only ferrous (Fe^{+2}) cations can be absorbed into apoferritin during reconstitution of ferritin (*19*). However once a partial core is present, a small amount of the Fe^{+3} can be exchanged back out of the core to transferrin (*19*). Therefore, ferric ions from the metal complex which are reduced in vivo can be incorporated into ferritin cores for storage. If these ferric ions are exchanged back to transferrin in the blood stream, the net effect would be higher levels of radioactivity in the blood than one would predict by pM values. The trivalent ferric and indium ions reduction potentials vary by an order of magnitude: ($E°[Fe^{+3} \rightarrow Fe^{+2}] = -0.036$; $E°[In^{+3} \rightarrow In^{+2}] = -0.49$). Indium is more difficult to reduce. In addition, the In^{+2} ion is unstable in aqueous media. $InCl_2$ (In_2Cl_4) decomposes to form the In^{+3} ion and a metallic indium precipitate (*20*). Thus, one cannot explain the high blood radioactivity for indium with the ferritin incorporation mechanism.

To investigate this possibility further, radioactivity levels in the liver were measured out to 2 hr postinjection. Fe[^{59}Fe]-meso-EHPG complexes show good uptake in the liver within 5 min postinjection, but very slight clearance over 2 hr. The liver has a high concentration of ferritin, and so this result supports the theory that the iron metal is incorporated into ferritin for storage. Any Fe[^{59}Fe]-meso-EHPG complex, which is not reduced, clears normally through the gut. The ^{68}Ga-meso-EHPG is kinetically stable against transferrin exchange and is not subject to metal reduction in the liver. Thus, this complex clears rapidly from the liver and through the gut. Other studies indicate that free In^{+3} metal ions in vivo are trapped in the liver (*12*). Since the ^{111}In-meso-EHPG is relatively unstable to transferrin exchange based upon pM, one would expect the free metal carried by transferrin to be deposited in the hepatocytes of the liver. This agrees with the result of very slow clearance of ^{111}In radioactivity from the liver. However, as in the case of iron complexes, any ^{111}In-meso-EHPG which survives in vivo catabolism and reaches the liver is cleared normally through the gut.

Based on pM, meso complexes produce more free metal ions through equilibrium than racemic complexes. Assuming free iron is more available for reduction to Fe^{+2}, one would predict higher liver uptake for Fe[^{59}Fe]-meso-EHPG than for Fe[^{59}Fe]-racemic-EHPG. In fact, the liver does show higher uptake of iron for the meso diastereomer. In-meso-EHPG has a lower pM value than In-racemic-EHPG. Since free ^{111}In cations are trapped by the liver, one would expect higher uptake in the liver for ^{111}In-meso-EHPG than ^{111}In-racemic-EHPG. Instead, just the opposite is true. A possible explanation involves more careful comparison of EHPG versus transferrin pM values.

In the cases of iron and gallium, pM values are significantly higher than those for transferrin. Presumably, a very small amount of free metal ions is available for transferrin binding. This is generated by release from the EHPG complex through equilibrium or catabolism. In contrast, In-EHPG pM values are lower than those of transferrin. A much greater number of metal ions are released from the complex. The variation from predicted liver uptake for indium complexes may reflect a kinetic effect of stereospecific catabolism or transferrin involvement in metal ion release which is not obvious from the small amount of metal ions which is released from iron or gallium.

Generally, large liver radioactivity levels correlate with increased lipophilic character (*12*). One can measure relative lipophilicity with HPLC. Longer retention times on reverse-phase HPLC indicate a greater degree of lipophilicity. Since the meso-EHPG free ligand is slower to elute than the racemic-EHPG free ligand, meso-EHPG is more lipophilic. All of the meso-EHPG metal complexes also have longer retention times on the column than the corresponding racemic-EHPG metal complexes. The effect of the metal on lipophilicity is not the same for both diastereomers. The meso-EHPG metal complex lipophilicity increases from gallium to indium to iron. Racemic-EHPG metal complex lipophilicity increases from gallium to iron to indium.

One can estimate the amount of radioactivity cleared through the liver by totaling the %ID/organ data in the liver, small intestine, and upper large intestine at 2 hr postinjection (Fig. 2). The resulting numbers indicate

an increase in percent clearance through the liver from iron (14.1% ± 4.4%) to indium (17.7% ± 2.4%) to gallium (39.7% ± 3.9%). This is a surprising result based upon our measurement of the lipophilicity of the complexes measured by HPLC. The order of increasing liver uptake is the exact reverse of the order of increasing lipophilicity for the meso diastereomer. This indicates that liver uptake is not solely a function of lipophilicity. Instead, other less obvious parameters govern the amount of radioactivity clearing through the liver.

This study suggests that the in vivo behavior of EHPG complexes is a function of their pM values and reduction potentials. Lauffer and Vincent (*21*) have reported that the clearance of substituted derivatives of Fe-EHPG is determined by stereospecific binding to bilirubin sites on human serum albumin. The results of our study indicate a simpler, less specific mechanism.

CONCLUSION

HPLC and in vivo studies indicate that the stereochemistry of a metal complex has important effects upon behavior. Significant differences in HPLC retention times of diastereomeric complexes are observed. In vivo studies show that meso-EHPG metal complexes of iron, gallium and indium clear the blood more rapidly than racemic-EHPG metal complexes. Stereospecific behavior is also observed in liver uptake. These results indicate that absolute configurations of chiral metal complexes significantly effect in vivo behavior.

The state of a metal-ligand equilibrium can be described with pM values or stability constants (log K). Blood clearance of EHPG metal complexes is best explained by comparing pM values of the EHPG metal complex with that of transferrin metal complexes.

Liver uptake of iron, gallium or indium EHPG metal complexes cannot be explained with measurements of lipophilicity. Instead, pM values and metal reduction potentials can be used to interpret liver uptake data. Metal cations which are easily reduced to stable ions such as Fe^{+3}, can present a complicated metabolism mechanism. Behavior of metal complexes of metals which are not reduced in vivo, such as Ga^{+3} and In^{+3}, can be explained with pM values and lipophilic character. Metal ion chemistry is an important parameter in explaining inorganic pharmaceutical chemistry. One cannot merely extrapolate data from one trivalent metal complex to a different metal with the same ligand.

ACKNOWLEDGMENTS

The authors wish to thank Mr. Michael Brown and Ms. Zoe Ann McKinnon for their technical assistance with the animal experiments. This work was supported in part by NIH grant CA-42925 and by DOE grant DE-FG02-87ER60512.

REFERENCES

1. Lauffer RB, Vincent AC, Padmanabhan S. Hepatobiliary MR contrast agents: 5-substituted iron-EHPG derivatives. *Magn Reson Med* 1987; 4:582–590.
2. Patch MG, Simolo KP, Carrano CJ. The cobalt (III), chromium (III), copper (II), and manganese (III) complexes of ethylenebis((*o*-hydroxy-phenyl)glycine): models for metallotransferrins. *Inorg Chem* 1982; 21:2972.
3. Frost AE, Freedman HH, Westerback SJ, et al. Chelating tendencies of N,N′-Ethylenebis-[2-(*o*-hydroxyphenyl)]-glycine. *J Am Chem Soc* 1958; 80:530–536.
4. Schroder KH. Complex formation with trivalent Iron and N,N′-Ethylene-bis-[2-(*o*-hydroxyphenyl)]glycine. *Nature* 1964; 202:898–899.
5. Schroder KH. A study of the polargraphic behavior of N,N′-Ethylenebis-[2-(*o*-hydroxyphenyl)]glycine in the presence of iron ions. *Acta Chem Scand* 1964; 3:596–602.
6. Anderegg G, L'Eplattenier F. Die Aciditatskonstanten und der Eisenkomplex des N,N′-Athylen-bis-*o*-hydroxyphenylglycins (-EHPG). *Helv Chim Acta* 1964; 47:1067.
7. Harris WR, Martell AE. Aqueous complexes of gallium. *Inorg Chem* 1976; 15:713–720.
8. Salama S, Richardson FS. Spectroscopic studies of lanthanide ion binding to multidentate ligands in aqueous solution. 2. N,N′-Bis(2-hydroxyphenyl)-ethylenedinitrilo-N,N′-diacetate. *Inorg Chem* 1980; 19:635–639.
9. Rajan KS, Mainer S, Rajan NL, et al. Studies on the chelation of aluminum for neurobiological applications. *J Inorg Biochem* 1981; 14:339–350.
10. Taliaferro CH, Motekaitis RJ, Martell AE. New multidentate ligands. 22. N,N′-Dipyroxyethylenediamine-N,N′-diacetic acid: a new chelating ligand for trivalent metal ions. *Inorg Chem* 1984; 23:1188–1192.
11. Bannochie CJ, Martell AE. Affinities of racemic and meso forms of N,N′-Ethylenebis[2-(*o*-hydroxyphenyl)glycine] for divalent and trivalent metal ions. *J Am Chem Soc* 1989; 111:4735–4742.
12. Mathias CJ, Sun Y, Welch MJ, et al. Targeting radiopharmaceuticals: comparative biodistribution studies of gallium and indium complexes of multidentate ligands. *Nucl Med Biol Int J Radiat Appl Instrum Part B* 1988; 15(1):69–81.
13. Hunt FC. Reversed-phase high-performance liquid chromatography of gallium-67 and indium-111 chelates of EHPG, HBED, and their derivatives. *Appl Radiat Isot* 1988; 39(4):349–352.
14. Harris WR, Pecoraro VL. Thermodynamic binding constants for gallium transferrin. *Biochemistry* 1983; 22:292–299.
15. Thakur ML. Gallium-67 and indium-111 radiopharmaceuticals. *Int J App Rad Isot* 1977; 28:183–201.
16. Martell AE, Motekaitis RJ. *Determination and use of stability constants.* New York: VCH Publishers, Inc.; 1988.
17. Green MA, Welch MJ, Mathias CJ, et al. Gallium-68-1,1,1-Tris(5-methoxysalicylaldiminomethyl)ethane: a potential tracer for evaluation of regional myocardial blood flow. *J Nucl Med* 1985; 26:170–180.
18. Harrison PM, Lilley TH. In: Loehr TM, ed. Ferritin. *Iron carriers and iron proteins.* New York: VCH Publishers, Inc.; 1989:123–238.
19. Treffry A, Harrison PM. The binding of ferric iron by ferritin. *Biochem J* 1979; 181:709.
20. Busev Al. *The analytical chemistry of indium.* Greaves JT, translator. New York: Pergamon Press; 1962.
21. Lauffer RB, Vincent AC. Stereospecific binding of rac-Iron (III), N,N′-Ethylenebis[(5-bromo-2-hydroxyphenyl)glycinate] to the bilirubin site on human serum albumin. *J Am Chem Soc* 1987; 109:2216–2218.
22. Aisen P, Liebman A, Zweier J. Stoichiometric and site characteristics of the binding of iron to human transferrin. *J Biol Chem* 1978; 253:1930–1937.