INDIUM LABELED PARTICLES FOR LUNG IMAGING: HOMOGENEOUS AND HETEROGENEOUS PHASE REACTIONS

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SUMMARY

The labeling of microspheres with ^{113m}In, intended as potential lung and capillary-bed imaging agents, was studied using an EDTA-exochelator to specifically bind indium. Incubation of the azo-phenyl-EDTA-microsphere with indium, resulted in an unstable preparation which primarily localized in the liver and spleen. When, however, ^{111}In was reacted with the diazo-phenyl-EDTA in solution, and this complex then coupled to the microspheres, excellent binding resulted. Similarly, complexing of ^{113m}In to azo-phenyl-EDTA-albumin in solution followed by aggregation, also yielded radiolabeled particles which were resistant to washing and localized over 90% in the lung, immediately upon injection to rats. These results have been rationalized by proposing a mechanism where the kinetics of homogeneous and heterogeneous phase reactions account for the nature of the different products formed. The present study, while it leads to better indium microspheres and aggregates than recorded in the literature, above all points out to the need of a better understanding of reaction kinetics in the preparation of such radiopharmaceuticals.

Key Words: Indium, particles, homogeneous/heterogeneous phase reactions

INTRODUCTION

There are numerous reports in the literature on attempts to develop an

efficient method for specific binding of ^{113m}In to particles that would localize in the lung capillaries (1-7). The problems involved in such preparations are the time of synthesis required, the purity of the final product, and the yield. Because none of such agents were entirely satisfactory for either perfusion or aerosol ventilation studies, we decided to re-examine the chemistry involved in the preparation of such indium complexes.

The chemistry of Indium that is of use in the preparation of radiopharmaceuticals is basically limited to chelation (complexing) processes in aqueous solutions. Neither native albumin, nor albumin modified by denaturation, has functional groups of sufficient complexing strength that will retain the indium label when it is in competition with more specific trivalent metal chelating agents, such as transferrin in blood. The proposal by Goodwin and Sundberg (8,9), that binding of an EDTA [ethylenediamine tetraacetic acid] residue as an "exochelate" to a protein would significantly increase the stability of the indium-protein chelate, led us to attempt to apply this principle to microspheres of albumin, and to study their biological behaviour.

EXPERIMENTAL

The human serum albumin (HSA) was a salt-poor, 25% solution (Hyland Labs.) The azo-compound was prepared according to Sundberg et al (9), as described below. The microspheres were obtained from the 3M Co. $^{111}InCl_3$ (Medi+Physics) and ^{113m}In was obtained from a Tin-Indium Generator (New England Nuclear).

Diazophenyl EDTA

A solution of the 1-(p-benzenediazonium)-EDTA("diazophenyl-EDTA") fluoborate, containing 5 μmoles/ml, was prepared as reported (9), with minor modification to increase stability. Concentration of the solution of 1-(p-aminophenyl)-EDTA, even at 5°C at reduced pressure (0.2mmHg) resulted in significant decomposition. This could be avoided by converting the amine into its fluoborate salt, prior to volume reduction.

A solution containing 0.115 mmoles p-(aminophenyl)-EDTA in 45 ml of water (prepared as directed by Sundberg (8) by Raney Ni reduction of the nitro-compound) is mixed with 0.5 ml of 50% fluoboric acid, at 5°C with continuous stirring. This solution can be stored at 5°C. The above solution was concen-

trated at reduced pressure and 5°C to 5 ml, and mixed, over 15 minutes in an ice bath, with 1.15 ml (0.115 mmoles) of 0.1M $NaNO_2$. The reaction was completed in 1 hr. Diazophenyl-EDTA-fluoborate, when stored at 5°C, is stable for 1-2 weeks.

Azo-EDTA-Microspheres

With constant stirring at 5°C, 3 ml of the above solution was added drop-wise to a suspension of 15 mg of HSA microspheres in 10 ml borate buffer, pH 9, containing 15 mg Tween 80 (polysorbate 80). The total addition time was 15 minutes, and the pH 9 was maintained by drop-wise addition of 0.2N NaOH. The reaction mixture was stirred for 24 hours at 5°C, after which it was adjusted to pH 7 with 0.2N HCl. The microspheres were centrifuged at 2000 rpm for 10 minutes and the supernatant removed and washed 3 times with 10 ml portion of cold, distilled water. The washed azo-EDTA microspheres were suspended in 15 ml distilled water, containing 15 mg Tween 80, and stored at 5°C. Microscopic examination of the azo-EDTA microspheres shows them to have slight yellow color, with a number of particles showing evidence of disruption.

Azo-EDTA-HSA

A solution of 1-(p-benzenediazonium)-EDTA fluoborate containing 12 μmoles/ml was prepared as described above with constant stirring at 5°C. 10 ml of this solution was added drop-wise to 10 ml of a 1% solution of HSA in 0.01M EDTA-0.12 M $NaHCO_3$ at pH 8.1. The pH was maintained by drop-wise addition of 0.3N NaOH. The reaction mixture was then stirred for another hour at 5°C. The azo-EDTA-HSA was purified by dialysis against 0.1M sodium citrate buffer, pH 6, at 5°C for 72 hours, with fresh buffer used every 24 hours, followed by dialysis against distilled water at 5°C. The final solution was 30 ml and was stored at 5°C.

^{113m}In-Azo-EDTA-Microspheres

With constant stirring, 1 ml of solution of $^{113m}InCl_3$ (1.7 mCi/ml) in 0.05N HCl, containing 0.1 ml of 1N acetic acid, was added to a suspension of 1 mg of azo-EDTA-microspheres in 1 ml acetate buffer, pH 6, containing 1 mg Tween 80. The mixture was adjusted to pH 4 with 0.2M Na_2HPO_4, and heated with stirring at 75°C for 15 minutes. The suspension was cooled,centrifuged, washed with acetate buffer, pH 7.2 and 0.9% NaCl, and resuspended in 0.9% NaCl at specific activity of 2 mCi/ml. Table 1 summarizes the activity distribution between the microspheres and the supernatant following these washings.

^{111}In-Azo-phenyl-EDTA microspheres

With constant stirring, 0.1 ml of 1N acetic acid is added to 1 ml of $^{111}InCl_3$ in 0.05N HCl, and this solution added to 1 ml (8 μmole) of Diazophenyl-EDTA fluoborate. After 5 minutes the pH was adjusted to pH 4 with 1N NaOH, and the solution was cooled to 5°C. The above solution was added, over a period of 15 minutes, drop-wise, to a stirred suspension, also maintained at 5°C, of 30 mg HSA microspheres in 5 ml borate buffer, pH 9, containing 10 mg Tween 80. The pH 9 was maintained by addition of 0.2N NaOH. The suspension was stirred at 5°C for 24 hours, the suspension then centrifuged and the supernatant decanted. The labeling yield was only 19%. The labeled microspheres were washed twice with 10 ml portions of acetate buffer, pH 7.25; 90% of the activity remained bound. The labeled microspheres were resuspended in 0.9% NaCl to a specific activity of 60 μCi/0.1 ml.

^{113m}In(^{111}In)-Azo-EDTA-HSA

2 ml azo-EDTA-HSA were added to 2 ml (3.5mCi) $^{113m}InCl_3$ in 0.05N HCl, containing 0.1 ml of 1N AcOH, and the mixture adjusted to pH 6 with 1N Na_2HPO_4. This mixture was subjected to TLC on silica gel in both water:ethanol:15N NH_4OH (125:50:0.1) or 0.1N HCl, and all radioactivity appeared associated with the albumin fraction.

^{113m}In(^{111}In)-Azo-EDTA-HSA Aggregates

The above solution of the ^{113m}In-Azo-EDTA-HSA was heated in a conical centrifuge tube at 100°C for 30 minutes with constant agitation and cooled in an ice bath. In some cases, 0.06 ml of a $Zr(SO_4)_2$ solution (30 mg/ml) was added before aggregation, as proposed by Chapman et al (7) with no improvement in the biological stability. The aggregated material was separated from the supernatant by centrifugation at 2500 rpm for 10 minutes, followed by 2 washings with 1 ml portions of cold (5°C) acetate buffer, pH 4. The aggregates were then suspended in 0.9% NaCl, and adjusted to the desired specific activity. Microscopic examination of particle size revealed no particles larger than 70 micron.

Stability Studies of Labeled Azo-EDTA-HSA-Aggregates

The relative stability of binding of the label to the azo-EDTA ligand on

either microspheres or aggregates was determined by washing the labeled particles with 0.01N HCl, acetate buffer pH 4 or 0.9% NaCl, followed by centrifugation. The supernatant and particles were counted and the results are presented in Table 1, as percentage of the original activity retained on the particles.

Animal Distribution Studies

Male Sprague-Dawley rats weighing 150-240 grams each were injected intravenously into the tail vein with 50 μCi each of test material and sacrificed by cerebral dislocation following heart-bleeding at 30, 60 and 120 minutes, as well as 4, 6 and 12 hours post injection. Lungs, spleen, pancreas, adrenals, kidneys, thyroid, testes and heart were weighed in toto and counted. The liver was weighed in toto and sampled. Blood, muscle and bone were sampled and counted. All samples were counted in a Beckman Biogamma Counter for one minute per sample. The results obtained are tabulated as organ/blood and % injected/gram.

RESULTS

Both chelated products, ^{113m}In-Azo-EDTA-microspheres and ^{113m}In-Azo-EDTA-macroaggregates were tested for their relative stability by washing with either 0.1N Acetate buffer of 0.01N HCl and centrifugation. The results, expressed as percent of activity retained on the particles are presented in Table 1. It is obvious from this table that while the labeled macroaggregates retained about 99% of their activity after repeated washings with acetate buffer, the labeled microspheres lose the label easily with remarkable dissociation:

TABLE 1. Percent Activity Retained On Particles Following Washings With Various Agents

Washing Solution	^{113m}In-Azo-EDTA Microspheres	^{113m}In-Azo-EDTA Macroaggregates
Labeling Solution	82%	98%
0.1N Acetate Buffer, pH 4, 1st Washing	72%	98%
0.1N Acetate Buffer, pH4, 2nd Washing	66%	98%
0.1N Acetate Buffer, pH 4, 3rd Washing	62%	99%
0.1N Acetate Buffer, pH4, 4th Washing	---	99%
0.01N HCl, 1st Washing	50%	88%
0.01N HCl, 2nd Washing	---	93%
0.9% NaCl	47%	98%

TABLE 2. Distribution of ^{113m}In - Chelated Macroaggregated Albumin In Male Sprague-Dawley Rats ½ - 12 h After IV Administration (n=10-12)

Organ/Blood Ratio is Given in Boldface

% Injected/Gram is Given in Italics

	½h	1h	2h	4h	6h	12h
Lung	965.4 ± 111.4	928.1 ± 113.3	708.3 ± 108.7	338.1 ± 29.8	231.1 ± 26.6	157.3 ± 16.0
	50.7 ± 7.2	*49.3 ± 6.0*	*41.7 ± 7.5*	*44.4 ± 4.6*	*28.7 ± 2.7*	*19.3 ± 2.4*
Liver	9.4 ± 1.3	10.6 ± 1.3	18.7 ± 2.5	27.1 ± 2.6	30.8 ± 2.2	55.7 ± 2.9
	0.5 ± 0.1	*0.8 ± 0.1*	*1.6 ± 0.4*	*3.6 ± 0.4*	*4.1 ± 0.5*	*6.8 ± 0.7*
Spleen	5.2 ± 1.0	8.0 ± 1.4	17.7 ± 2.4	35.8 ± 4.4	48.2 ± 5.8	88.5 ± 8.3
	0.3 ± 0.1	*0.6 ± 0.1*	*1.6 ± 0.4*	*4.8 ± 0.6*	*6.3 ± 0.9*	*11.1 ± 1.6*
Pancreas	0.5 ± 0.1	0.4 ± 0.0	0.6 ± 0.2	0.5 ± 0.1	0.6 ± 0.1	1.1 ± 0.4
Adrenals	0.9 ± 0.3	0.7 ± 0.1	1.1 ± 0.2	1.8 ± 0.6	2.6 ± 0.7	14.7 ± 6.8
Kidneys	1.5 ± 0.2	1.3 ± 0.1	1.9 ± 0.1	2.2 ± 0.2	2.5 ± 0.2	4.1 ± 0.2
Thyroid	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Testes	0.3 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.4 ± 0.2	0.3 ± 0.2	0.5 ± 0.4
Muscle	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.2
Bone	0.4 ± 0.1	0.4 ± 0.0	0.7 ± 0.0	1.0 ± 0.2	1.3 ± 0.3	3.8 ± 2.0
Heart	1.3 ± 0.2	1.3 ± 0.4	1.2 ± 0.2	0.8 ± 0.1	0.7 ± 0.1	0.6 ± 0.4

TABLE 3. Distribution (organ/blood ratio) of ^{111}In-Chelated Macroaggregated Albumin and (^{111}In) ^{113m}In-Chelated Microspheres in Male Sprague-Dawley Rats at Various Time Intervals After IV Administration (n=8-10)

	^{111}In-Chelated Macroaggregated Albumin				^{111}In-Microspheres	^{113m}In-Microspheres
	½h	1h	2h	6h	1h	1h
Lung	7.28 ± 0.76	9.56 ± 1.49	13.16 ± 2.27	14.31 ± 3.53	349.11 ± 12.89	91.92 ± 12.04
Liver	3.60 ± 0.96	7.72 ± 2.08	8.98 ± 2.57	17.88 ± 4.77	0.77 ± 0.03	287.17 ± 37.82
Spleen	2.97 ± 1.04	6.36 ± 1.84	10.95 ± 4.06	22.73 ± 7.99	0.35 ± 0.02	101.86 ± 18.83
Pancreas	0.23 ± 0.01	0.42 ± 0.11	0.48 ± 0.09	0.77 ± 0.15	- - -	- - -
Kidneys	1.28 ± 0.04	1.93 ± 0.31	2.28 ± 0.23	3.38 ± 0.39	3.60 ± 0.44	2.90 ± 0.36
Adrenals	0.45 ± 0.06	0.88 ± 0.25	0.84 ± 0.27	1.41 ± 0.42	1.99 ± 0.11	0.98 ± 0.12
Thyroid	3.13 ± 0.58	3.66 ± 0.69	2.85 ± 0.41	3.07 ± 0.73	3.95 ± 1.26	1.24 ± 0.46
Testes	0.07 ± 0.01	0.11 ± 0.01	0.21 ± 0.01	0.31 ± 0.02	0.23 ± 0.06	0.14 ± 0.03
Muscle	0.07 ± 0.01	0.13 ± 0.02	0.15 ± 0.03	0.21 ± 0.03	0.27 ± 0.08	0.25 ± 0.03
Bone	0.27 ± 0.05	0.64 ± 0.15	0.88 ± 0.32	1.74 ± 0.58	0.95 ± 0.15	2.10 ± 0.59
Heart	0.39 ± 0.02	0.57 ± 0.14	0.65 ± 0.13	0.67 ± 0.09	0.38 ± 0.01	0.32 ± 0.02

The results of the ^{113m}In-Azo-EDTA-MAA distribution studies are presented as organ/blood ratios and for 3 organs as percent injected/gram tissue (Table 2), up to 12 hours after injection of the agent. For the other organs listed on page 2, the percent injected/gram of tissue remained low (<0.2%/g) and did not change with time. Similar distribution studies are also given for ^{111}In-Azo-EDTA-MAA, up to 6 hours post injection (Table 3). Previous distribution studies of the Azo-phenyl-EDTA microspheres chelates to both isotopes were also carried out, and some of their representative runs are summarized in Table 3.

DISCUSSION

The limited chemical stability of the indium labeled microspheres to repeated washings with both buffers and solution of 0.01N HCl suggests that the indium does not appear to be strongly bound to the azo-EDTA moiety on the microspheres. In addition, the data on biological distribution of such indium (^{113m}In)-labeled microspheres in rats clearly parallel those that are characteristic of the distribution of a colloid, e.g. high liver and spleen uptake. In addition, significant physical instability of the microspheres was observed when they were exposed to the coupling conditions.

Welch (10) had analyzed the chemistry of carrier-free indium in solution and suggested that two factors should be taken into account in the formation and stability of indium complexes: the kinetics of formation and the stability constant of the final complex.

In applying these concepts to the preparation of the Indium-azo-phenyl-EDTA microspheres, we also relied on the acetate complex (I) intermediate proposed by Welch (10). In this system, the indium ion eluted in 0.05N HCl is first converted to the acetate complex, and then reacted, at pH 7, with the azo-phenyl-EDTA microspheres. While the formation of an indium-EDTA complex in solution is rapid and complete, in the system described by Welch, the same does not appear to be the case in this work.

These findings can be rationalized by assuming that, in the formation step, there is a competition between hydroxide formation (II) and formation of the

EDTA complex (III). When the azo-phenyl-EDTA is in homogeneous phase and the transfer of indium from the acetate complex to the azo-phenyl-EDTA complex proceeds rapidly, e.g. $k_3 > k_2$, whereas when the reaction proceeds in a heterogeneous phase the rate of the reaction of the EDTA moiety with indium is much slower, e.g. $k_2 > k_3$, and formation of the hydroxide is favored:

$$In^{+++} + 3Ac^- \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} InAc_3 \qquad (I)^*$$

$$InA_3 + 3OH^- \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} In(OH)_3 + 3Ac^- \qquad (II)^*$$

$$InAc_3 + EDTA \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} In\text{-}EDTA + 3Ac^- \qquad (III)^*$$

Obviously, each equation is a composite of many steps. (11)
It is likely that a two body collision between the small indium acetate and the large azo-phenyl-EDTA-microsphere is kinetically less favorable than the collision between the indium acetate complex and the hydroxide ion, both of similar size and therefore more diffusible than the large microsphere. Such a sequence of events would lead to the formation of an indium hydroxide, which would remain in a colloidal form and be stabilized by deposition on the surface of the particulate matter.

This mixture of a colloid and a particle will re-dissociate in the body into a radioactive colloid, which separates itself from the particle and, as any colloid will, give a high liver uptake. Both the chemical data, which suggests significant solubility of the ^{113m}In when the microsphere are treated with the 0.05N HCl, and the biological data, showing high liver uptake, are consistent with the formation of a colloid on the microsphere surface.

If, however, the azo-phenyl-EDTA residue is coupled to a soluble protein such as albumin, the formation kinetics are reversed and it is indeed the ^{113m}In-azo-phenyl-EDTA-albumin complex that is formed in preference to the colloid (8,9). Based on these concepts, it was logical to prepare the azo-phenyl-EDTA-indium-albumin complex in homogeneous phase, followed then by

aggregation of the albumin and formation of a macroaggregate. The chemical stability data are given in Table 1, and exposure of the complex to 0.05N HCl did not result in significant washout of indium activity. This suggests the presence of a stable complex. The data on the biological distribution in rats of this indium-azo-phenyl-EDTA-MAA is given in Table 2, and show that more than 95% of the injected activity is localized in the lung. The presence of less than 0.5% of the activity in the liver or spleen suggests near total absence of any colloid, and therefore absence of reaction II.

Additional support for the unfavorable kinetics of the heterogeneous phase reaction is offered by the positive biological results attained (see last column, Table 3) when indium is complexed to the azo-phenyl-EDTA in solution, prior to coupling, and the chelate is coupled subsequently to the microsphere. However, while this method yields a suitable labeled microsphere, and supports the postulated reaction mechanism, the product is of little significance as a potential lung scanning agent because of the long preparation time required and the relatively long lived radionuclide required therefore. This preparation could, however, be of interest to physiological studies in animals where a microsphere labeled with a longer lived radionuclide might be desirable.

The relative stability of the complex poses a different problem. When the indium-azo-phenyl-EDTA-MAA was prepared with ^{111}In and the complex utilized several hours (10-15) after formation, results from animal distribution data showed significant liver uptakes, although the chemical stability data obtained immediately after product preparation was good (Table 1). When we switched to ^{113m}In and were forced to utilize the short-lived radiopharmaceutical agent immediately after preparation, very satisfactory results, as given in Table 2, became apparent. In addition, a few hours post-injection, the relative activity localized in the lung decreases, with concomitant liver and spleen involvement. This suggests biological degradation of the indium macro-aggregate complex, with indium liberated both in a colloid and possibly a transferrin bound form. The lack of stability of the final EDTA azo-albumin complex is somewhat more difficult to understand, but it may be related both to the steric strain of the molecule and the reactivity of the EDTA-In complex on the particulate surface.

From a kinetic point of view, hydroxide formation is a slow process and while its stability constant is lower than that of EDTA alone, it is possible that the steric strain of the aggregates results in a significantly lower stability constant for the indium-azo-phenyl-EDTA-MAA complex. This may result in the indium undergoing a slow rearrangement to the more stable indium hydroxide.

In conclusion, the problems discussed here of the chemistry and biochemistry of an indium complex suggest that more extensive chemical and biological studies of these and related systems need to be performed to gain a better understanding of the mechanism involved, and to develop consistent indium labeled radiopharmaceuticals for clinical use.

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