

Studies on the Metabolic Fate of ¹¹¹In-labeled Antibodies

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Indium-111-labeled antibodies, though providing superior photon flux to iodine-labeled antibodies, can exhibit high levels of accumulation in some non-target organs. In an effort to understand the nature of this non-target uptake we have evaluated the molecular weight of ¹¹¹In species retained in several tissues by radio-FPLC (sizing chromatography) following injection of [¹¹¹In]DTPA 5G6.4, a murine monoclonal antibody, into normal mice. Blood, liver and kidneys were removed, and liver and kidneys were homogenized at several time points after antibody injection. The proportion of ¹¹¹In-containing species was found to vary with the tissue and with time. Analysis of blood showed only radiolabeled antibody. In the liver, several ¹¹¹In species were identified with molecular weights compatible with intact antibody, [¹¹¹In]transferrin, and low molecular weight complexes, with an increase in the proportion of [¹¹¹In]transferrin and low molecular weight species occurring over time. While the same molecular weight species were also identified in the kidneys, the kidneys contained the largest percentage of low molecular weight species which increased over time. When ¹²⁵I-labeled 5G6.4 was injected and the tissues similarly analyzed, only radioactive material with the molecular weight of intact antibody was detected. Comparison of two methods of purification of [¹¹¹In]labeled antibody after labeling revealed a significant difference in the organ uptake of radiolabeled products for ¹¹¹In. Although dialysis was sufficient for the removal of labile ¹¹¹In, as determined by TLC, subsequent sizing chromatography on Bio-Gel P-60 dramatically dropped the hepatic and renal uptake of ¹¹¹In relative to blood and diminished the proportion of the low molecular weight species present on sizing FPLC of extracts from tissues. These data indicate that low and intermediate molecular weight ¹¹¹In compounds are accreted in the liver and kidneys following the i.p. injection of ¹¹¹In-labeled monoclonal antibodies and that their uptake can be diminished by more stringent radioantibody purification. This knowledge may be valuable in developing methods for reducing non-target ¹¹¹In uptake.

Introduction

Indium-111-labeled monoclonal antibodies have shown promise in the area of cancer detection. Unfortunately, their use to date has been limited somewhat by the high background levels observed in several non-target organs, particularly the liver (Halpern *et al.*, 1983; Sands and Jones, 1987). While increasing the dose of unlabeled antibody protein may diminish the hepatic ¹¹¹In uptake, and suggests, a saturable phenomena, this approach is not fully effective (Otsuka and Welch, 1985; Patt *et al.*, 1988). Improvement in the efficacy of monoclonal antibodies for imaging and therapy of cancer would seem to rely on reducing the non-target background of radioantibody (Goodwin, 1987).

Several explanations have been put forth regarding the mechanism for hepatic uptake of ¹¹¹In attached to antibodies. Initially, it was felt possible that denatured antibody was being removed by the

reticuloendothelial cells in the liver (Khaw *et al.*, 1980). A second possible explanation relies on the chemical similarity of In³⁺ to Fe³⁺. The possibility exists that ¹¹¹In is incorporated into the transport and storage system of iron and thereby shuttled to the liver by transferrin for storage in the cellular protein, ferritin (Sands and Jones, 1987). A third possibility is that antibodies localize in the liver regardless of the radiolabel (Halpern *et al.*, 1983). This last possibility is supported by recent work which identifies Fc receptors in hepatocytes as well as studies with antibody fragments demonstrating less absolute and relative hepatic antibody accretion than with intact IgG (Sancho *et al.*, 1984; Wahl *et al.*, 1983). The fact that high liver backgrounds are not observed with an iodinated antibody is presumed to be due to rapid dehalogenation in the liver. The work of Sands and Jones (1987) strongly suggests that the site of ¹¹¹In uptake in the liver is within the hepatocytes.

This study was undertaken to provide additional insight regarding the accumulation in the liver observed for ¹¹¹In-labeled antibodies. Our approach was

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to examine the radiolabeled species in blood, and in extracts from the liver and kidneys by sizing chromatography (FPLC) after injection of [^{111}In]5G6.4, a murine IgG2a monoclonal antibody (Wahl *et al.*, 1986). Non-tumor-bearing mice were used to rule out possible contributions to hepatic uptake arising from the formation and hepatic accretion of immune complexes. The ability of the 5G6.4 antibody to localize to ovarian tumors has been addressed in other work (Wahl *et al.*, 1988). We have also compared two methods of purification of the radiolabeled antibody, the effect these methods had on tissue distribution, and the FPLC profile of ^{111}In species extracted from the sites of visceral accumulation, as well as those seen for the ^{125}I control.

Methods

A murine monoclonal antibody, 5G6.4 (IgG2a), grown in mouse ascites and purified by Staphylococcal protein A chromatography (Ey *et al.*, 1978), was coupled to DTPA using literature methods (Hnatowich *et al.*, 1983) with small modifications. A fresh solution of cDTPA (diethylenetriaminepentaacetic dianhydride), the bicyclic anhydride of DTPA (Sigma), was prepared for each label in anhydrous diethyl ether and protected from moisture (10–16 mg cDTPA/100 mL ether). A suspension containing 10 eq of cDTPA was transferred to an Eppendorf bullet tube and the solvent was removed by vacuum. An equivalent amount of the murine antibody (16 mg/mL) in 0.05 M NaHCO_3 (pH 7.0–7.5) was added and the solution was allowed to react for 1–2 h. Free DTPA was then removed by centrifugation using a centrifugal concentrating membrane (Centricon-Amicon Inc.). During this step, the buffer was also changed to 0.1 M citrate, pH 5. The coupled protein solution retained in the Centricon tube was rinsed with three aliquots of citrate buffer and finally restored to a workable volume (150–250 μL) in citrate. Indium-111 chloride of high specific activity (NEN) was then transferred directly into the antibody solution. The pH adjustment of NEN with an acetate buffer (pH 6) reported by Hnatowich was avoided because of the uncertain solubility of indium chloride in acetate solution (Cotton and Wilkinson, 1980) and at high pH (Moeller, 1941; Moerlein and Welch, 1981). By transferring the coupled antibody to citrate at pH 5 this step was unnecessary.

These conditions resulted in an average of 1–2 chelates per protein molecule. TLC (Gelman ITLC SG sheets) in 0.1 M citrate showed the incorporation of ^{111}In to be >95%, as assessed by automated gel scanning (Bioscan). The labeled protein was subsequently dialyzed for 24 h vs 2–3 changes of citrate (pH 5) to remove free radiolabel. Half of this material was then chromatographed on a Bio-Gel P-60 (Bio-Rad) sizing column, 8 \times 1 cm. Recovery of radioactivity ranged from 76 to 3%. An equal quantity of 5G6.4 was also labeled with ^{125}I by the Iodogen[®]

method (Fraker and Speck, 1978). (1.25 mg antibody protein was reacted with 3.5 mCi Na^{125}I , using 1/8 ($\mu\text{g}/\mu\text{g}$) Iodogen/protein ratio). The radiolabeled protein was then dialyzed and one-half of this material was also chromatographed, on the P-60 sizing column. Dialyzed only (1–3 mCi/mL, 2–3 mg/mL) or dialyzed and chromatographed (0.4–0.7 mCi/mL, 0.5 mg/mL) radioantibody were used in the animal studies after the samples were adjusted to pH 7.

Examination of the radiolabeled components in each animal tissue by FPLC was carried out in normal BALB/c mice which had received approx. 50 μCi of labeled antibody by i.p. injection. Animals were sacrificed at varying time points and blood samples were allowed to clot with analyses performed on serum. Liver and kidneys were excised and homogenized on ice with mortar and pestle in a saline solution containing the following protease inhibitors: pepstatin (2 μM), leupeptin (2 μM), bacitracin (0.1 mg/ml), phenylmethylsulfonyl fluoride (1 mM) and aprotinin (1 TIU/mL). Following centrifugation, sizing analyses of the supernatant were carried out immediately on a Pharmacia FPLC system with a Superose 12 gel filtration column. Fractions were collected and counted on a Packard γ -counter. The percentage recovery of radioactivity from these manipulations was difficult to determine accurately, but appeared to be much higher for serum (~80%) than for liver and kidneys (~20%), in part due to incomplete homogenation. Protein assignments were tentatively made by comparison of elution times with authentic samples. Positive identifications were made by concentrating the pertinent fractions followed by gel electrophoresis and Coomassie Blue staining.

To compare the influence of the final antibody purification on biodistribution, normal BALB/c mice were injected i.p. with the radiolabeled proteins (16 $\mu\text{g}/\text{animal}$, 9–14 μCi). For each radioisotope there were two sets of mice (6–8 animals per set): one group received the dialyzed antibody while the second group received the dialyzed and chromatographed portion. Administration of the dose was by i.p. injection for ease of delivery and to minimize stress on the animals. Previous work has indicated no significant difference in tissue distribution as a result of i.p. vs i.v. injection at delayed times of sample acquisition (Wahl *et al.*, 1988). The mice were sacrificed after 48 h and tissues were collected, weighed, and counted. Statistical analysis was by the Student's *t*-test.

Results

Figure 1 shows that the distribution of ^{111}In -labeled species was found to vary with the sampled tissue. The activity profile for blood revealed that only the intact antibody was present at each time point [Fig. 1(a, b)]. The activity profiles for liver [Fig. 1(c, d)] and kidneys [Fig. 1(e, f)] indicated that

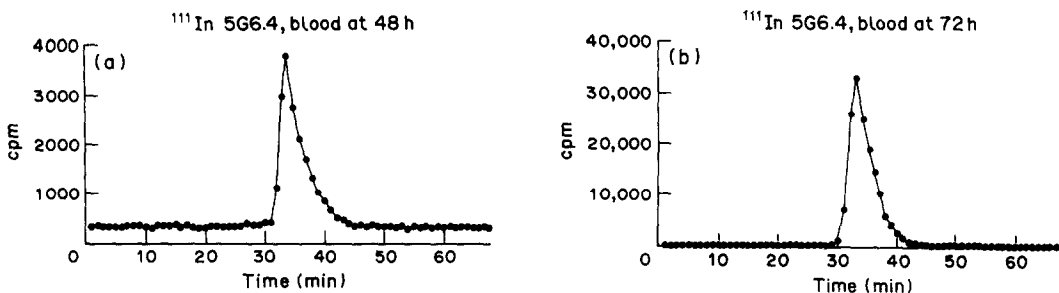


Fig. 1(a, b). Only [^{111}In]5G6.4 at 34 min is observed in the serum by FPLC radio chromatography at 48 and 72 h after antibody injection.

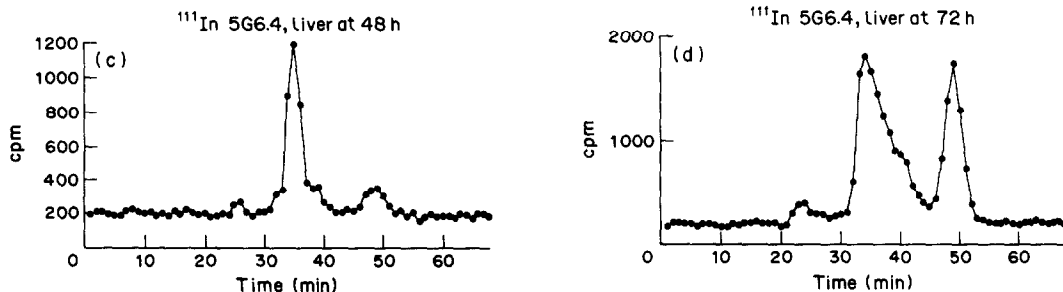


Fig. 1(c, d). Activity profile of ^{111}In -containing species in extracts from the liver at 48 and 72 h. Note the increase in the low molecular weight (49–50 min) and transferrin molecular weight (38–40 min) species at the later time point.

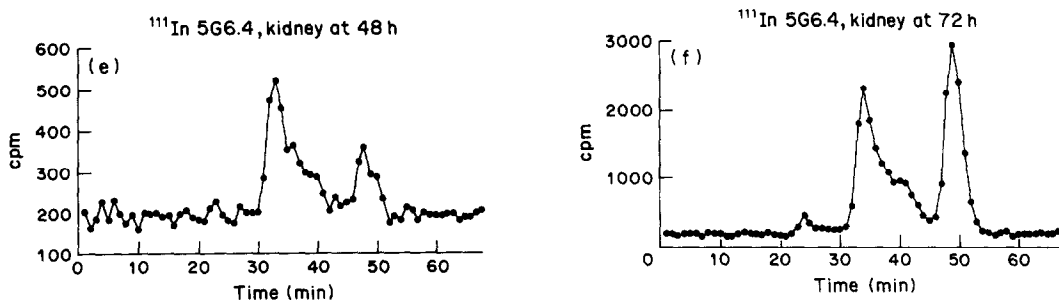


Fig. 1(e, f). Renal extract activity profiles: in addition to the labeled antibody at 34 min, [^{111}In]transferrin at 38–40 min and low molecular weight species at 49–50 min accumulate in the kidneys as a function of time. Note the large quantity of the low molecular weight species particularly at the later time point. A small amount of insoluble material is also detected in the void volume at 24 min. This is also the case in the liver extract.

at least three ^{111}In -containing species were present. In addition to the intact antibody which eluted at 34 min, an ^{111}In species with a molecular weight most compatible with binding to transferrin was observed at 40 min, while a low molecular weight species was found at 49 min. The time course of FPLC profiles clearly illustrated that these latter two components increased with time in the liver [Fig. 1(c, d)] and kidney [Fig. (e, f)] relative to the concentration of intact antibody. A small amount of insoluble material, a fourth peak, was also detected in the void volume at 24 min. Comparison to the iodinated antibody pointed out that the accumulation of radiolabeled species in these organs was considerably different from that of ^{111}In . The data obtained for [^{125}I]5G6.4, shown in Fig. 2(a–c), demonstrated the

uniform single profile of intact antibody in each tissue.

Purity is one of the most important factors which can affect the performance of a radiolabeled antibody. Uncomplexed ^{111}In , or ^{111}In loosely associated with the antibody, should quickly be complexed by serum transferrin and perhaps carried to the liver for storage in ferritin. To address the possibility that ^{111}In -containing species which localize in non-target organs are impurities injected along with the antibody (rather than catabolic products), we have compared two methods of purification and the subsequent effect of biodistribution.

The 5G6.4 antibody was again labeled with ^{111}In (under the conditions described previously) and dialyzed against citrate buffer to remove labile ^{111}In .

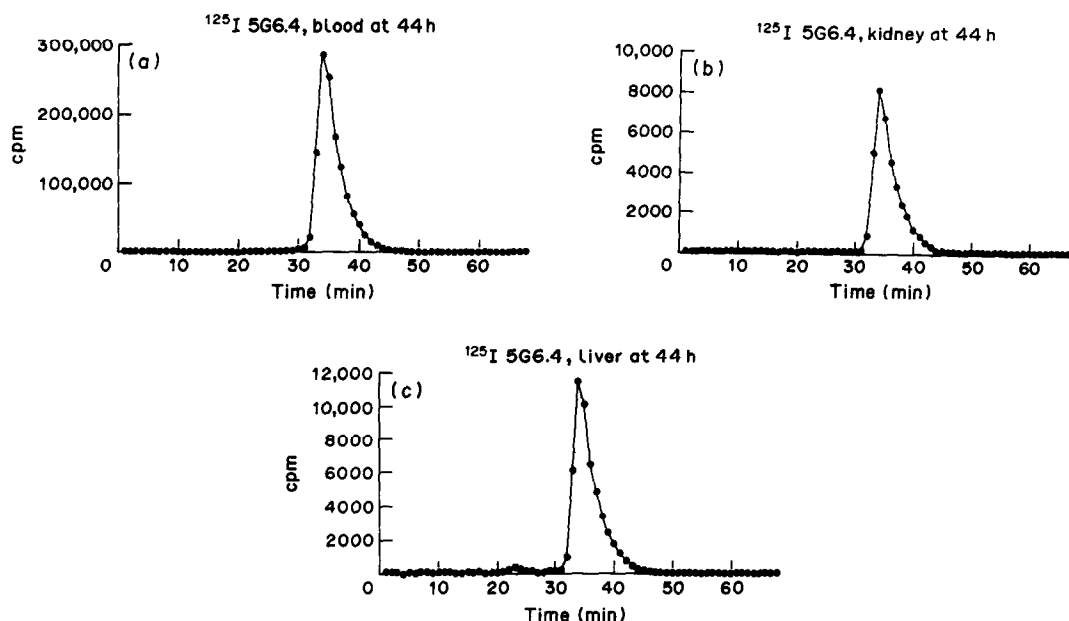


Fig. 2(a-c). In contrast to ^{111}In , catabolism of the ^{125}I -labeled protein does not result in the accumulation of secondary ^{125}I -labeled metabolic products in the liver and kidneys. Blood, liver and kidneys all exhibit a single peak profile corresponding in molecular weight to [^{125}I]5G6.4.

TLC of the labeled protein prior to dialysis showed 3% of the radioactivity was not bound to the protein; the radiochromatogram is shown in Fig. 3. After dialysis, <1% of the radioactivity was in the labile form in this buffer system. This material was then divided into two aliquots and one aliquot was chromatographed on Bio-Gel P-60 (gel filtration chromatography). The radiochromatogram appeared unchanged by chromatography vs dialysis alone, as can be seen in Fig. 3. The biodistributions of the two preparations were then compared in BALB/c mice. The activity profiles from liver extracts are displayed in Fig. 3 and indicate that a greater fraction of the radioactivity measured corresponded to intact antibody for the chromatographed sample. These differences were found at both time points, suggesting that a significant difference existed between the two samples. Of particular interest was the fact that this difference, seen as the *in vivo* distribution, was not apparent by TLC of the original preparation.

To further substantiate these results, additional studies were performed in a larger population of

6–8 mice per group. The animals were sacrificed 48 h after the i.p. injection of [^{111}In]DTPA 5G6.4 and tissues were weighed and counted. Once again the results indicated important differences between the two methods of purification. Table 1 shows that similar levels of radioactivity were found in liver and kidneys for both groups, but the values for blood were substantially different ($P < 0.01$), with correspondingly higher liver/blood and kidney/blood ratios for the dialyzed-only preparation.

To determine if a component of loose, non-covalent, association of ^{111}In with the antibody might be responsible for this difference in the *in vivo* biodistribution, the 5G6.4 antibody was labeled with ^{125}I and purified in a similar manner. Two groups of mice received comparable doses of protein and radioactivity purified by dialysis or dialysis with FPLC, and were sacrificed after 48 h. Indeed, the values listed in Table 1, particularly the virtually identical liver/blood and kidney/blood ratios, indicate no meaningful difference exists between the two groups.

Table 1. Biodistribution of [^{111}In]DTPA 5G6.4 and [^{125}I]5G6.4

	Liver*	Kidney*	Blood*	Liver/blood*	Kidney/blood*
[^{111}In]DTPA 5G6.4					
Dialysis	0.174 (0.068)	0.233 (0.065)	0.130 (0.056)	1.493 (0.873)	1.894 (0.354)
Dialysis + P-60	0.162 (0.017)	0.255 (0.022)	0.223 (0.057)	0.763 (0.174)†	1.21 (0.307)†
[^{125}I]5G6.4					
Dialysis	0.064 (0.033)	0.065 (0.031)	0.254 (0.119)	0.255 (0.065)	0.258 (0.046)
Dialysis + P-60	0.083 (0.029)	0.075 (0.026)	0.264 (0.099)	0.322 (0.047)	0.294 (0.059)

Two days post i.p. injection.

*Units of %ID/kg/g. Data are given as mean (SD).

†Significantly different at $P < 0.01$.

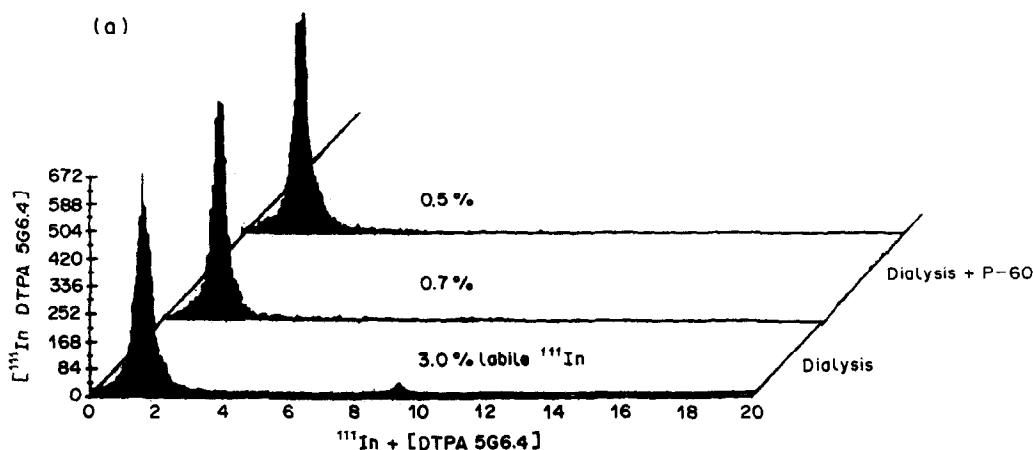


Fig. 3(a). [^{111}In]DTPA 5G6.4 was first purified by dialysis, then a portion was further purified by P-60 chromatography. The count profiles of the TLC chromatograms for the labeled protein before and after each purification step are shown. Protein bound ^{111}In is found near the origin, while labile ^{111}In is observed between 8 and 10 cm. The preparations all are nearly devoid of labile ^{111}In .

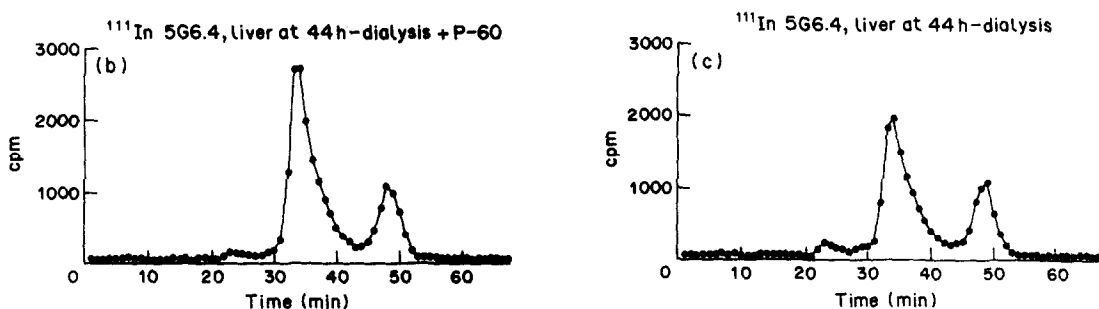


Fig. 3(b, c). The activity profiles of ^{111}In -containing species extracted from the livers at 44 h following i.p. injection of each of two methods of purification, dialysis or dialysis + P-60 FPLC, are shown. Although no difference is detected by TLC between the ^{111}In -labeled antibody prepared by the two purification procedures, a smaller percentage of low molecular weight species was found in the liver for the P-60 fraction at 20 h (not shown) as well as 44 h post sacrifice.

Discussion

The presence (on FPLC analysis) of ^{111}In species compatible with transferrin and low molecular weight complexes in liver and kidneys, and their absence in the blood, suggests several possibilities. These components could represent products of catabolism which has occurred in these organs, or they might correspond to impurities which were injected along with the antibody and cleared from circulation. Alternatively, both mechanisms could simultaneously contribute to the observed accumulation. It should be noted that even if the injected antibody is free from impurities, transcomplexation from the ^{111}In -DTPA-antibody complex to transferrin is known to occur (Hnatowich *et al.*, 1985). The presence of these species also indicates that the high renal and hepatic background activity observed upon imaging is not simply due to the vascularity of these organs, but rather to the accumulation of these secondary ^{111}In products. Although similar in component parts, the FPLC profiles of extracts from kidneys, in comparison to liver, displayed a higher fraction of low molecular weight species at each time point. Quite

possibly this low molecular weight form(s) of ^{111}In was available for excretion. These findings are in considerable contrast to those for [^{125}I]5G6.4 where only the intact antibody is seen. This indicates that iodinated impurities or catabolic by-products were not present or stored in the liver and kidney tissues. Of course, metabolic products may be present but not detectable by our methods if deiodination is occurring and iodine is rapidly excreted.

The higher level of radioactivity found in blood for the P-60 chromatographed sample, together with the information obtained from the activity profiles, implies that a higher percentage of the injected radioactive dose corresponded to tightly-labeled intact antibody in the P-60 sample. These data suggest that impurities were present in the sample which was dialyzed but not chromatographed, and that these impurities were rapidly removed from the blood, with resulting lower blood radioactivity levels. An important consequence of this is seen in the organ/blood ratios listed in Table 1. For the sample which was dialyzed only, the normal tissue/blood ratios are significantly higher and would thus result in a much higher non-target background upon imaging. A

second implication of these data, which should also facilitate imaging, is that the higher blood levels in the P-60 purified sample should allow for higher tumor exposure to antibody and presumably increase antibody uptake by tumor.

The differences observed for these two ^{111}In samples rely on the additional purification accomplished by interaction with the Bio-Gel P-60 matrix. However, analysis of the radiolabeled protein by TLC and FPLC indicated only one component prior to chromatography, ^{111}In -labeled antibody. A plausible explanation for the differential biodistribution is that electrostatic interaction between In^{3+} and trace carboxyl residues in the P-60 chromatographic gel removes metal ions which are loosely associated with the antibody protein. This type of non-specific radio-metal attachment to the antibody would provide a source of ^{111}In which would be easily dissociated *in vivo* and accreted in the liver and kidneys. Secondary interactions between charged species and molecular sieving gels have been recognized by others (Jacobs, 1977; Janson, 1967, 1978; Reeves *et al.*, 1970). If this phenomenon does arise from interaction between a positively-charged metal ion and carboxyl groups within the gel, chromatography should have no effect upon the biodistribution of an iodinated antibody since iodide possesses a negative charge and is covalently coupled. Our results (Table 1) support this hypothesis.

In this work, the biodistribution of [^{111}In]DTPA-5G6.4 was modified by chromatography on a sizing gel, Bio-Gel P-60. Based on the activity profiles, the difference in blood values (listed in Table 1) is attributed to a difference in the antibody concentration. We conclude that the remainder of the original injected dose for the sample which was not chromatographed corresponded to ^{111}In which is weakly held to the protein. This purification procedure should provide a two-fold benefit to imaging. First, liver/blood ratios were significantly lower after chromatography, indicating a lower liver uptake background. Secondly, blood levels were higher which directly affects delivery of the antibody to tumor. Finally, we note that TLC was not a useful indicator for label purity in this regard. These observations expand upon those of Esteban *et al.* (1987) and Meares *et al.* (1984) regarding the necessity of careful purification of ^{111}In -labeled antibody, and suggest that methods lacking an FPLC step may well lead to high visceral background activity. Indeed, Esteban has shown that HPLC purification of ^{111}In antibody further enhances *in vivo* behavior. In this study the presence of labile ^{111}In was deduced only by comparison of the tissue distribution results and by examination of the activity profiles in the extracted tissue. In conclusion, lower molecular species containing ^{111}In accumulate in the liver and kidneys following radioantibody injection. Their accretion can be reduced by additional purification procedures.

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