Protein Binding Studies of Technetium-99m-Labeled Phosphine and Isocyanide Cationic Complexes

Giuseppe D. Zanelli, Neil Cook, Avijit Lahiri, David Ellison, Peter Webbon, and Gillian Woolley

Divisions of Radioisotopes and Clinical Sciences, Clinical Research Centre; Department of Cardiology, Northwick Park Hospital, Harrow, Middlesex; and Royal Veterinary School, North Mymms, Herts, UK

Most ^{99m}Tc/phosphine/isocyanide complexes synthesized to date show preferential uptake by the myocardium of many animal species but not in man. A new complex has been synthesized, $[^{99m}Tc(DEPE)_2(CNR)_2]^+$(DEPIC), where R = t − butyl isocyanide, which in three animal species images the myocardium very well, but in humans it remains primarily in the blood pool. One reason for the difference in the behavior of these complexes in different species could be the characteristics of their binding to plasma proteins. The protein binding characteristics of DEPIC and two other well-known complexes have therefore been studied. Whereas the other complexes bind nonspecifically to many proteins both in animal and human plasma, DEPIC binds almost exclusively to prealbumin in humans but nonspecifically to other proteins in the rabbit. The binding sites in human plasma appear to be those normally occupied by thyroxine on the prealbumin tetramer and these can be blocked by sodium salicylate.

J Nucl Med 29:62–67, 1988

In 1981, Deutsch and his colleagues showed that cationic complexes of technetium-99m (^{99m}Tc) such as $[^{99m}Tc(diars)_2X_2]^+$ (*1*) and $[^{99m}Tc(dmpe)_2(X)_2]^+$, where X_2 is Cl or Br (*2*), could be used to image the myocardium in animals. It soon became apparent, however, that these complexes, including all the variations produced by using different ligands and halide counterions (*3*) failed to localize in the human myocardium. More recently, Holman et al. (*4*) have synthesized several cationic [^{99m}Tc]hexakis(alkyl-isonitrile) complexes and found that although these did show pronounced myocardial uptake in both animals and humans there was still a considerable inter-species difference in their pharmacokinetic behavior. This point has been highlighted very recently by Lahiri and his colleagues (*5*), who showed that a [^{99m}Tc]cationic complex containing both phosphine and isocyanide ligands, which gave excellent images of the myocardium in rats, dogs and especially in the rabbit, when tested in humans was found to remain in the blood pool. This complex has in fact been proposed by the present authors as an efficient one-injection alternative to labeled red cells for radionuclide ventriculography (*5*). Preliminary routine tests showed that the above complex was totally bound to the plasma proteins in both human and rabbit plasma. But more detailed tests showed that the type of protein to which the complex became attached was not the same in human and rabbit plasma. The protein binding characteristics of these complexes therefore appeared to be at least a factor in determining their behavior in the animal body and may point to the kind of molecular structure and size best suited for targeting in a given tissue. With this in mind it we decided to investigate the protein binding characteristics in human and rabbit plasma of three representative variations of the complexes described above, whose behavior in both humans and animals was known. The three complexes investigated were (A) $[^{99m}Tc(DEPE)_2(Cl)_2]^+$ (B) $[^{99m}Tc(DEPE)_2(t\text{-}Bu\text{-}CN)_2]^+$ and (C) $[^{99m}Tc(t\text{-}Bu\text{-}CN)_6]^+$.

Received Feb. 9, 1987; revision accepted Sept. 14, 1987.

For reprints contact: G.D. Zanelli, MD, Radioisotopes Division, Clinical Research Centre, Watford Rd., Harrow, Middlesex HA1 3UJ, United Kingdom.

MATERIALS AND METHODS

Preparation of the Complexes

The complexes A and C were synthesized as described in the literature (*1,2*) with slight modifications as introduced by Radfar (*3*). In general, the preparation involves refluxing in ethanol/saline a mixture of ^{99m}Tc and the ligand(s) under nitrogen in the presence of a reducing agent ($Na_2S_2O_4$). For complex (B) the following sealed vial method was developed. The required activity of $^{99m}TcO_4$ in saline (1.0 ml), 0.3 mg of EGTA (ethylene glycol bis-(-aminoethyl ether)-N,N,N′,N′-tetraacetic acid),* and 50 μl of a 10 mg/ml ethanol solution of trans-dichloro bis(1,2-diethyl phosphino ethane)-iron ($[Fe(DEPE)_2Cl_2] + FeCl_4^-$]), prepared by the method of Radfar (*3*), were added to a 10-ml glass vial which was then capped, flushed with nitrogen and heated at 100° C for 15 min. The vial was cooled at room temperature (RT) for 5 min after which 0.5 ml of 0.2*M* HCl, 0.5 ml of 30 mg/ml of freshly prepared $Na_2S_2O_4 \cdot 2H_2O$* in saline and 0.5 ml of 20 mg/ml para-aminobenzoic acid† in saline were added and the vial left at RT for a further 30 min. Then 0.5 ml of 0.2*M* NaOH and 0.1 ml of 0.9*M* t-butyl isocyanide‡ in ethanol were added and the vial heated at 100° C for a further 5 minutes.

All complexes were cleared of insoluble materials by passing through a 0.22/μm filter.§

The purity of all complexes was checked by isocratic reverse phase HPLC using a Hamilton PRP-1 column (25 × 0.5 cm), eluted with acetonitrile: 0.1*M* ammonium acetate, pH 6.8, 65:35, at a flow rate of 1 ml/min. Under these conditions the complexes had retention times of 8.5 min (A), 11.5 min (B) and 43 min (C). The cationic nature of the complexes was determined by electrophoresis on 3 MM Paper strips, at 300V in 0.1*M* phosphate buffer pH 8.6.

Protein Binding

A 30 × 1 cm glass/Teflon column was packed with Sephadex G-75 beads¶ and equilibrated with 0.9% NaCl. Then either 0.1 ml of one of the complexes, or 0.2 ml of a mixture of a complex and human or rabbit plasma, or 0.1 ml (5 μCi) of ^{125}I-HSA** was placed on top of the gel, the column was eluted with 0.9% NaCl and fractions of 1.0 ml collected and counted for ^{125}I and ^{99m}Tc activity.

Blocking of the thyroxine binding sites on prealbumin (see later) was accomplished by adding 10 μl of 0.1*M* sodium salicylate* solution in saline to human plasma before mixing with the complex and equilibrating and eluting the same column with 0.1*M* sodium salicylate/saline at pH 7.2.

Molecular weight determinations were done on a 150 × 5 cm glass column filled with S-200 gel†† and eluted with 50 m*M* TRIS/10 m*M* NaCl buffer at pH 7.4. The column was calibrated using several standard molecular weight markers in the range 12.4 kDa (cytochrome C)* to 2,000 kDa (blue dextran 2000)¶ to obtain a Ve/Vo calibration curve.

Zone electrophoresis was carried out in 8% polyacrylamide gels†† by the method of Neville (*7*) in the absence of detergent at a constant 135 volts for 5 hr. The gels were stained with Coomassie blue-G 250.†† One lane, cut into 2-mm slices, was used for ^{99m}Tc activity profile determination using an automatic NaI crystal gamma-counter.‡‡

Animal Toxicology

Although no adverse reactions to [^{99m}Tc]phosphine or to [^{99m}Tc]isocyanide complexes have been reported in the literature (apart from a transient taste of chives), it was thought prudent to inject some rats with the equivalent of a human dose before undertaking the human studies. Five male Sprague-Dawley rats weighing 250–280 g were injected intravenously with 1 mCi each of DEPIC containing the quantities of all ingredients as specified above under *Preparation of the Complexes*. The rats were observed for signs of behavioral changes during the first two days after injection. On the third day they were killed using Sagatal Forte i.p.,§§ the principal organs were removed and after examination for gross abnormalities they were sent for histologic examination. White cell, red cell, and platelet counts were also performed.

Animal and Human Studies

The animals used were white male NZ rabbits weighing ~ 2.5 kg, Sprague-Dawley rats weighing 250–300 g and 15 kg mongrel dogs. The complexes were injected i.v. in the anesthetized animals and a Nuclear Enterprises Scinticamera V¶¶ was used to image the chest and/or whole body.

The human studies were carried out in volunteers after prior ethical and ARSAC (Administration of Radioactive Substances Survey Committee, the official body which licenses the use of radiopharmaceuticals in humans in the UK) committee permission. For the distribution studies ~74 MBq of the complexes were injected into an antecubital vein and

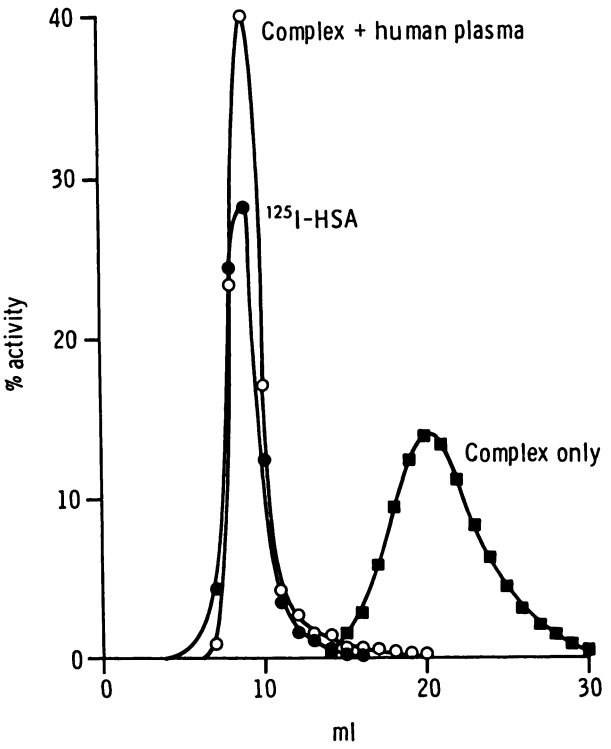

FIGURE 1
Elution patterns on Sephadex G-75 of complex B alone (■—■), complex B + human plasma, (○—○), and [^{125}I] HSA, (●—●) Elution with saline.

dynamic imaging over the chest area was carried out for 1 hr using an IGE 400 AT gamma camera.[***] Whole-body images were also acquired and the distributions of radioactivity were used to estimate whole-body radiation doses.

RESULTS

Protein Binding Characteristics of the Complexes

Figure 1 shows a typical elution pattern of complex B alone, complex/human plasma mixture and [^{125}I] HSA on Sephadex G-75/saline. All the complexes and mixtures of complexes with human or rabbit plasma give identical elution patterns showing binding to some molecular species large enough to be excluded from the G-75 gel (>40 kDa). Figures 2, 3, and 4 show the electrophoretograms and ^{99m}Tc activity profiles of mixtures of the complexes with human (A) or rabbit (B)

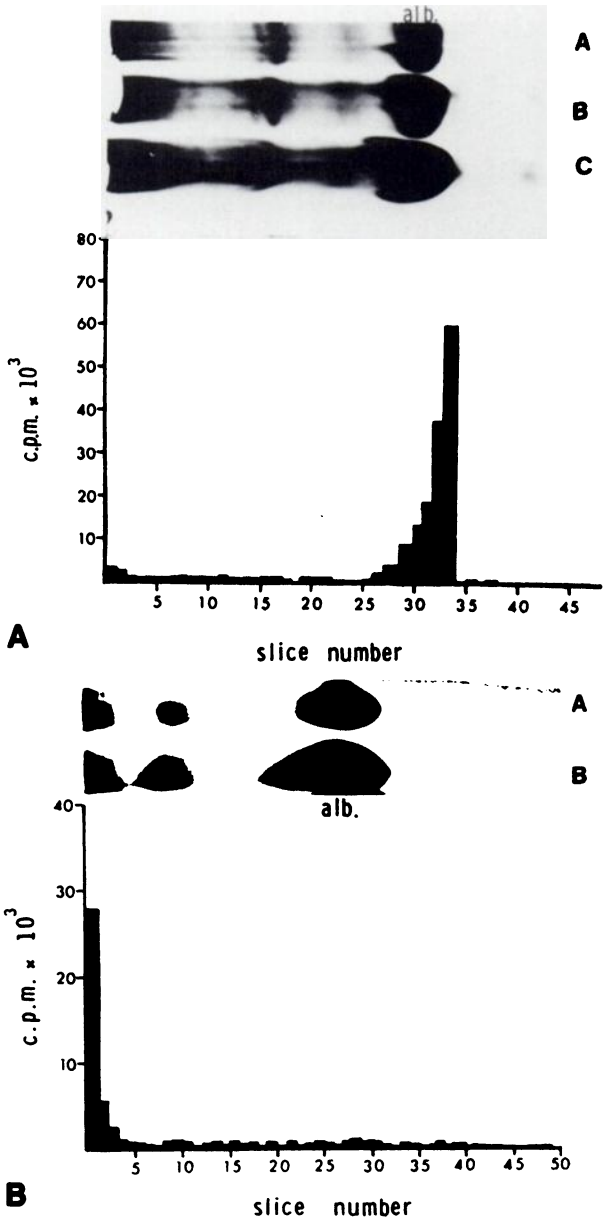

FIGURE 2
Electrophoretic and radioactivity profiles of $[^{99m}Tc(DEPE)_2(CL)_2]^+$ mixed with human plasma (A) and rabbit plasma (B).

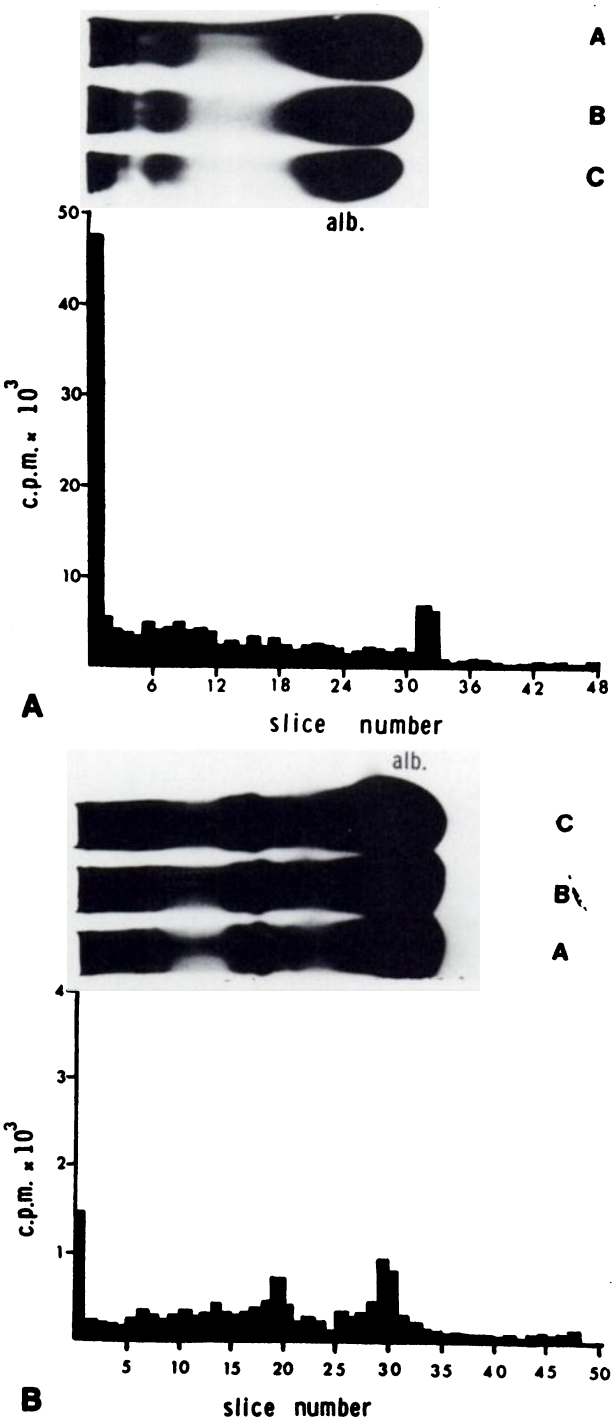

FIGURE 3
Electrophoretic and radioactivity profiles of $[^{99m}Tc(t\text{-}Bu\text{-}CN)_6]^+$ mixed with human plasma (A) and rabbit plasma (B).

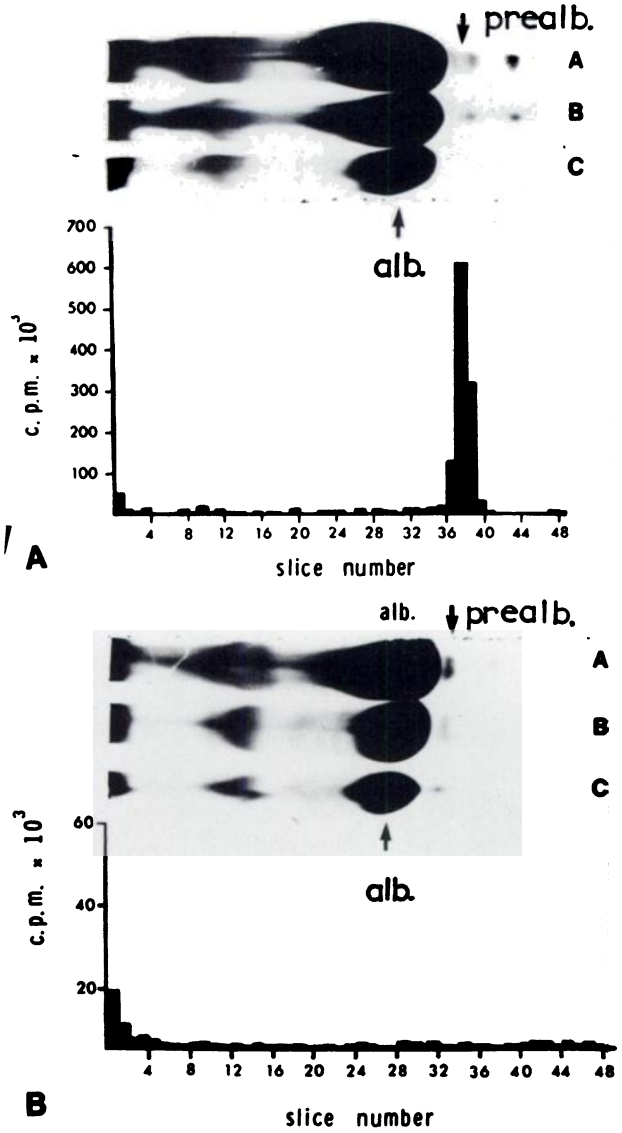

FIGURE 4
Electrophoretic and radioactivity profiles of $[^{99m}Tc(DEPE)_2(t\text{-}Bu\text{-}CN)_2]^+$ mixed with human plasma (A) and rabbit plasma (B).

plasma. $[^{99m}Tc(DEPE)_2CL_2]^+$ (Fig. 2) appears to be bound principally to albumin in human plasma and to large macromolecules in rabbit plasma. The hexakis complex $[^{99m}Tc(t\text{-}Bu\text{-}CN)_6]^+$ (Fig. 3) instead seems to be bound mainly to the very large globulins which, under the electrophoretic conditions used, remain at or near the top of the gel slab. With the rabbit plasma (Fig. 3B) there is also considerable nonspecific binding to smaller molecular weight proteins. Figure 4 shows the results for complex B, $[^{99m}Tc(DEPE)_2(t\text{-}Bu\text{-}CN)_2]^+$. The activity profiles for this complex in rabbit and human plasma are entirely different. In rabbit plasma (Fig. 4B) the complex shows nonspecific binding to a large spectrum of proteins. In the human plasma (Fig. 4A) all the activity is associated with a species which runs in front of the main albumin band but which is well stained by Coomassie-blue. This electrophoretic region has been shown (*8*) to be associated with two proteins: prealbumin (retinol-binding protein, 55 kDa) and alpha-acid glyoprotein (44.1 kDa). However, the S-200 gel filtration studies showed that all the radioactivity was associated with an entity of molecular weight about 57 kDa which is very near that reported for prealbumin (*9*). It is therefore very likely that in humans complex B is specifically bound to prealbumin.

Prealbumin is known to bind up to four molecules of retinol and also binds strongly two molecules of thyroxine (*10*). The thyroxine binding sites of prealbumin are thought to be within the central lumen of its four identical subunits (*11*) and have been reported to be blocked by sodium salicylate (*6*). Figure 5 shows the results of G-75 chromatography of a mixture of human plasma/sodium salicylate/complex B (elution with 0.1*M* sodium salicylate). It is clear that most of the complex is prevented from binding to the proteins and elutes with a Ve similar to that of the free complex. It is very likely therefore that this compound occupies the same binding sites as thyroxine on the prealbumin tetramer.

In rabbits, prealbumin is very likely to be a dimer as in rodents (*8*) and it is interesting that rabbit plasma prealbumin does not appear to bind complex B.

Animal Toxicology

None of the five rats tested showed any reaction to the injection of the large amounts of DEPIC used (~200 times the amount given to a 70 kg human). There were no significant variations in cell counts and no evidence

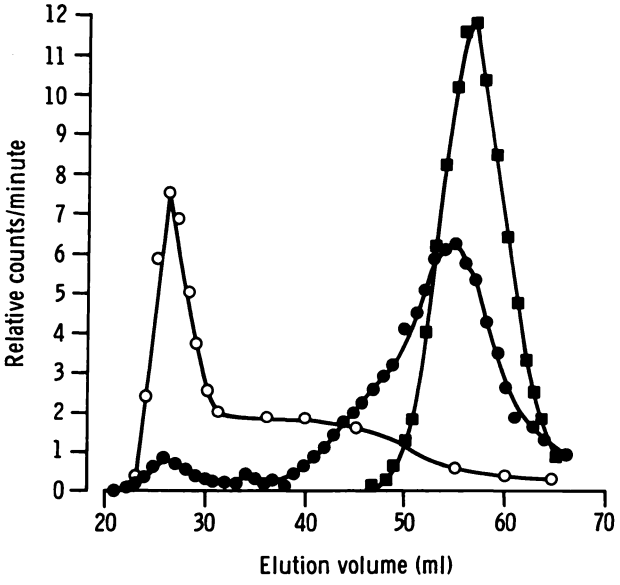

FIGURE 5
Elution patterns on sephadex G-75 of complex B alone (■—■) complex B + human plasma (○—○), and complex B + sodium salicylate + human plasma (●—●), elution with 0.1*M* sodium salicylate.

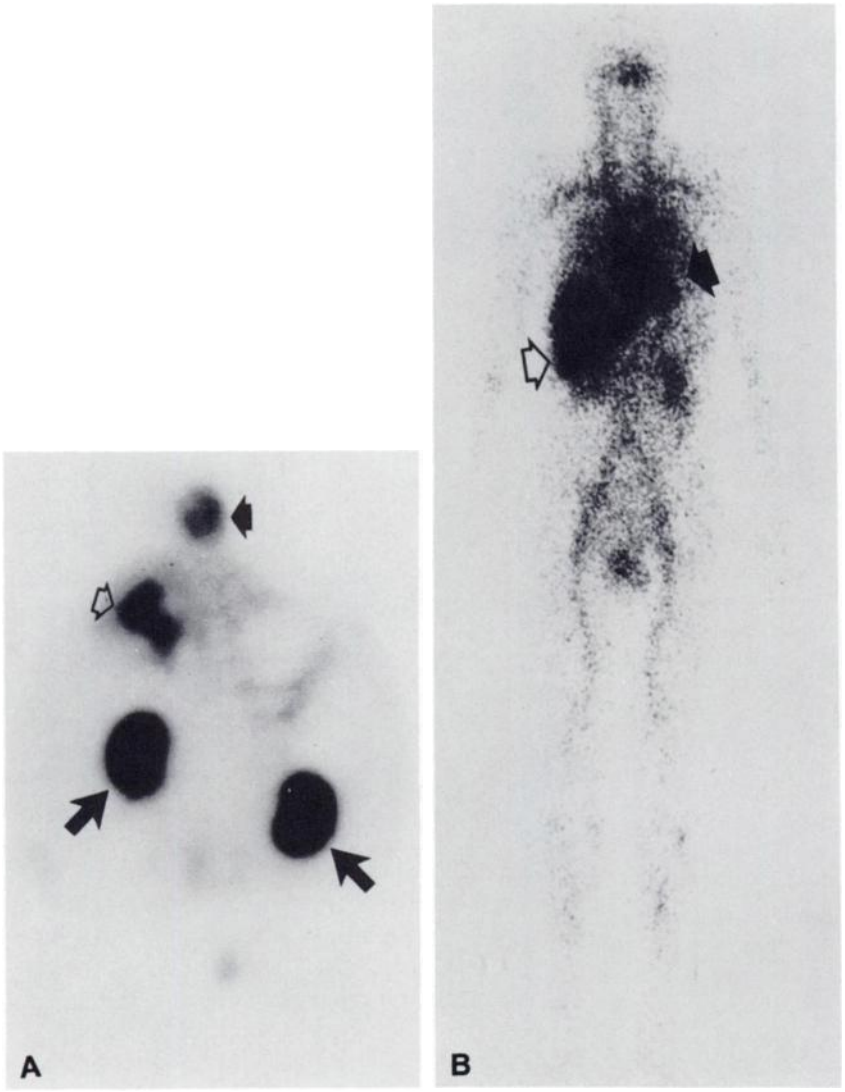

FIGURE 6
Scintigrams obtained 30 min after i.v. injection of complex B in a rabbit (A) and human volunteer (B). Heart activity indicated by closed arrowhead and gallbladder activity by open arrowhead. Arrows on (A) point to renal activity.

of pathology in any organ either on gross visual examination or on the histologic sections.

Animal and Human Studies

The imaging properties of complexes A and C in animals and humans have been already reported (*1–4*) and need not be shown again. Complex A shows good and persistent myocardial uptake in dogs, but in humans there is only a very transient myocardial uptake with most of the activity showing up in the liver. Complex C has good myocardium imaging characteristics in all species, but it too shows intense liver uptake. The distribution of complex B in rabbits and humans is shown in Figure 6. Figure 6A shows that in the rabbit the complex images the myocardium very well. The liver activity is rapidly ($T_{½}$ = 25 min) cleared via the gall bladder, leaving a clear picture of the heart muscle that lasts for several hours after injection. In humans instead (Fig. 6B) the complex images the blood pool.

Radiation Dosimetry

The model used for the dosimetry calculations has which bind easily to receptors at unwanted sites (*13*).

been fully described elsewhere (*14*). The results are shown in Table 1. The effective whole-body dose is higher for DEPIC than for labeled red cells (standard MUGA test) but not greatly so [0.0126 mSv MBq^{-1}, against 0.007 mSv MBq^{-1} (*15*)].

DISCUSSION

A survey of the existing literature makes it apparent that in the search for a ^{99m}Tc-labeled, ^{201}Tl substitute for myocardial imaging the main thrust has been to find a "potassium analog". That is, a cationic species which, like potassium, is actively transported in and out of the myocytes. It is becoming increasingly apparent, however, that the structure-distribution relationship of (radio) pharmaceuticals depends not on one but on several parameters (*12*). Thus, for example, attempts at increasing the lipophilicity of a substance by the addition of methyl groups may indeed lead to its increased extravasation, but may also lead to steric forms which bind easily to receptors at unwanted sites (*13*).

TABLE 1
Dosimetry of Complex B in Normal Male Subjects

Target organ	Dose	
	mrad/μCi	mGy/MBq
Testes	0.0455	0.0123
Breast	0.0106	0.0029
Red marrow	0.0222	0.0060
Lung	0.0224	0.0060
Thyroid	0.0084	0.0023
Bone surfaces	0.0084	0.0023
Gall bladder	0.1404	0.0379
ULI wall	0.1244	0.0336
LLI wall	0.0885	0.0239
SI wall	0.0719	0.0194
Bladder wall (3.5 hr void)	0.0406	0.0110
Liver	0.0342	0.0093
Effective dose equivalent	0.0468	0.0126

The work reported in the present communication shows that protein binding also plays an important part in the in vivo distribution of radiopharmaceuticals. The results of the present investigation show that in both human and rabbit plasma all complexes are bound to plasma proteins (Figs. 1–4). The difference is that whereas complexes A and C are bound either to albumin (A) or unspecifically to a variety of proteins (C), complex B is specifically bound to human prealbumin but unspecifically bound to several rabbit plasma proteins. Furthermore, complex B appears to bind to specific binding sites in tetrameric human prealbumin: the thyroxine binding sites. The strong bonds formed at such receptor sites mean that the bound molecule is unlikely to be released at equipotent sites in tissues. This would explain the tendency for complex B to remain in the blood pool in humans.

In the rabbit, complex B does not bind specifically to any protein but only unspecifically to a variety of proteins (Fig. 4B). Such binding is unlikely to be very strong and on reaching sites of higher affinity in tissues (e.g., the myocardium) the complex is released and behaves as a good myocardial imaging agent (Fig. 6A).

The class of cationic complexes examined (Tc-phosphine, Tc-isocyanide and Tc-phosphine-isocyanide) does indeed appear to have an affinity for the myocardium. Of the three complexes examined in the present investigation, the mixed Tc-phosphine-isocyanide complex appears to give the clearest myocardial images in the rabbit, mainly because of the rapid liver clearance. The results reported above would indicate that if specific binding to human plasma proteins could be prevented by minor structural modifications, the molecular configuration represented by complex B may point the way to the sought for thallium substitute for myocardial imaging.

NOTES

* Sigma Chemical Co., Poole, UK.
† PABA, Aldrich Chemical Co., Gillingham, UK.
‡ Fluka AG, Glossop, UK.
§ Millex FG, Millipore, UK Ltd., Harrow, UK.
¶ Pharmacia, Uppsala, Sweden.
** Amersham International plc, UK.
†† Bio-Rad, Watford, UK.
‡‡ (Gamma 7000 counter) Beckman Instruments.
§§ May & Baker Ltd., UK.
¶¶ Nuclear Enterprises, Reading, UK.
*** International General Electric Co., Slough, UK.

REFERENCES

1. Deutsch E, Bushong W, Glavan KA, et al. Heart imaging with cationic complexes of technetium. *Science* 1981; 214:85–86.
2. Deutsch E, Glavan KA, Sodd VJ, et al. Cationic Tc-99m complexes as potential myocardial imaging agents. *J Nucl Med* 1981; 22:897–907.
3. Radfar G, Brady F, Zanelli GD, et al. Cationic technetium-99m phosphine complexes as potential myocardial imaging agents. *Nucl Med Commun* 1984; 5:238.
4. Holman BL, Jones AG, Lister-James J, et al. A new Tc-99m labelled myocardial imaging agent, hexakis (t-butylisonitrile) technetium(1) [Tc-99m TBI]: initial experience in the human. *J Nucl Med* 1984; 25:1350–1355.
5. Lahiri A, Zanelli GD, Patel NM, et al. A ^{99m}Tc-labelled phosphine for radionuclide angiography in man. *Nucl Med Commun* 1986; 7:321–322.
6. Radfar G. Studies of cationic technetium complexes with potential in nuclear medicine. MSc Thesis, The University of London, England, 1984.
7. Neville DM Jr. Molecular weight determination of protein-dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system. *Biol Chem* 1971; 246:632–634.
8. Putnam FW. Perspectives—past, present and future. In: Putnam FW, ed. The plasma proteins, Vol. I. New York: Academic Press, 1975: pp 2–51.
9. Heide K, Haupt H, Schwick HG. Plasma protein fractionation. In: Putnam FW, ed. The plasma proteins, Vol. III. New York: Academic Press, 1977:545–588.
10. Irace G, Edelhoch H. Thyroxine-induced conformational changes in prealbumin. *Biochem* 1978; 17:5729–5733.
11. Ferguson RN, Edelhoch H, Saroff HA, et al. Negative cooperativity in the binding of thyroxine to human serum prealbumin. *Biochem* 1975; 14:282–289.
12. Coats EA. Quantitative structure-activity relationships. In: Spencer RP, ed. Radiopharmaceuticals, structure-activity relationships. New York: Grune and Stratton, 1981:3–22.
13. Katzenellenbogen JA, Carlson KE, Heiman DF, et al. Receptor binding as a basis for radiopharmaceutical design. In: Spencer RP, ed. Radiopharmaceuticals, structure-activity relationships. New York: Grune and Stratton, 1981:23–86.