

# Species-Dependent Binding of Copper(II) *Bis*(Thiosemicarbazone) Radiopharmaceuticals to Serum Albumin

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Copper-62-labeled pyruvaldehyde *bis*(N<sup>4</sup>-methylthiosemicarbazono)-copper(II) (Cu-PTSM) is a generator-based PET radiopharmaceutical under investigation for use in evaluation of tissue perfusion. Despite promising results from animals, problems have been encountered in the use of <sup>62</sup>Cu-PTSM to quantitate myocardial perfusion in humans at high flow rates, possibly due to species-dependent interactions of the tracer with serum albumin. **Methods:** Ultrafiltration and plasma/erythrocyte partitioning studies were performed to assess the protein binding of <sup>67</sup>Cu-labeled Cu-PTSM and six related copper(II) *bis*(thiosemicarbazone) complexes. **Results:** These studies reveal significant interspecies variability in the strength of Cu-PTSM binding to serum albumin, with <sup>67</sup>Cu-PTSM binding much more strongly to human albumin than to dog albumin. Most of the related Cu(II)-*bis*(thiosemicarbazone) complexes examined exhibit interspecies variability of albumin binding similar to that observed with Cu-PTSM. Two such complexes, Cu-ETS and Cu-*n*-PrTS, however, were identified that exhibit no preferential association with human serum albumin. **Conclusion:** Copper-62-PTSM exhibits substantial interspecies variability in the strength of its binding to serum albumin, which appears to explain the problems encountered in using animal data to predict <sup>62</sup>Cu-PTSM behavior in humans. The <sup>62</sup>Cu-ETS and <sup>62</sup>Cu-*n*-PrTS complexes may be viable alternatives to <sup>62</sup>Cu-PTSM for PET studies to evaluate quantitatively myocardial blood flow in humans.

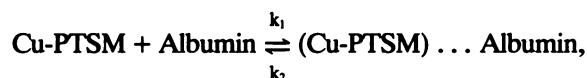
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Copper-62-labeled pyruvaldehyde *bis*(N<sup>4</sup>-methylthiosemicarbazono)-copper(II), Cu-PTSM (Fig. 1) has shown considerable promise as a generator-based multi-organ PET perfusion tracer (1–9). This compound is attractive for use in the study of cerebral and myocardial perfusion because, in addition to providing high first-pass extraction following intravenous injection, Cu-PTSM also

affords prolonged microsphere-like tissue trapping of the copper radiolabel (2–5). By using relatively simple blood analysis technique to quantitate <sup>62</sup>Cu-PTSM in arterial blood (10), myocardial perfusion in dogs can be quantified with <sup>62</sup>Cu-PTSM and PET over a wide range of flows (from 0.0–6.0 ml · min<sup>−1</sup> · g<sup>−1</sup>) (6).

PET studies by our group (2,6,11) and by others (8,9,12–14) have also shown <sup>62</sup>Cu-PTSM provides high quality images of the human heart at rest that qualitatively and quantitatively map the pattern of myocardial perfusion. In addition, <sup>62</sup>Cu-PTSM is a sufficiently sensitive tracer of cerebral blood flow (CBF) to allow mapping of focal CBF changes occurring in response to neurological stimulation (2,7). Recent studies by Bergmann et al. (11), as well as a number of independent studies of <sup>62</sup>Cu-PTSM (9,12–14), show that there is marked attenuation of Cu-PTSM myocardial uptake in humans at high flow rates. This is contrary to previous experience in dogs (6). The disparity between tracer behavior in dogs and humans appears to stem from substantial interspecies variability in the binding of Cu-PTSM to serum albumin (Eqs. 1 and 2) that effectively reduces the diffusibility of Cu-PTSM in human plasma:



Eq. 1

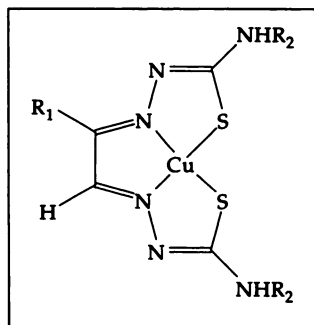
$$K = \frac{k_1}{k_2} = \frac{[(\text{Cu-PTSM}) \dots \text{Albumin}]}{[\text{Cu-PTSM}] [\text{Albumin}]}. \quad \text{Eq. 2}$$

We report results of an investigation of interspecies variability in Cu-PTSM binding to serum albumin along with albumin binding results for a number of related copper(II) *bis*(thiosemicarbazone) complexes (Table 1), which reveal this interspecies variability in albumin binding is highly compound-specific.

## MATERIALS AND METHODS

Thiosemicarbazone ligands were prepared as described previously (15). Copper-67-chloride was obtained from Brookhaven National Laboratory (Upton, NY) and Los Alamos National Laboratory (Los Alamos, NM). Human serum albumin (HSA; essen-

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**FIGURE 1.** General structural formula of the copper(II) bis(thiosemicarbazone) complexes investigated. For the parent compound, Cu-PTSM,  $R_1 = R_2 = \text{CH}_3$ .

tially globulin and fatty acid free) and dog serum albumin (DSA; essentially fatty acid free) were purchased from Sigma Chemical Company (St. Louis, MO) and used without further purification. Solutions of serum albumin were prepared fresh for each experiment at 35 mg/ml normal saline and refrigerated when not in use. All experiments involving animals were carried out following protocols approved by the Purdue Animal Care and Use Committee. Canine blood was drawn from mongrel dogs through a peripheral vein. Human blood was drawn from the antecubital vein of the principal investigator (MAG). In all cases, the blood was collected on the day of the experiment and anticoagulated with preservative-free heparin (10 U/ml). Whole blood was transferred immediately to a polypropylene tube and stored on ice until ready for use. Plasma was separated from whole blood after centrifugation at  $12,000 \times g$  for 1 min. Measurements of tracer partitioning between the plasma and red blood cell (RBC) fractions of whole blood were performed as described previously (10) using both dog and human blood. Rat biodistribution studies were performed as described previously (10,15) following injection of tracer ( $\sim 2 \mu\text{Ci}$  in 0.2 ml) into the femoral vein of ether-anesthetized animals. In the rat studies involving administration of  $^{67}\text{Cu}$ -PTSM in dog or human albumin solutions, the composition of the injectate was 35 mg albumin/ml saline containing 0.7% ethanol.

### Radioisotope Synthesis

The  $^{67}\text{Cu}$ -bis(thiosemicarbazone) complexes were prepared by evaporating the  $^{67}\text{Cu}$ -HCl solution to dryness with heating under a stream of nitrogen and then reconstituting in 0.25 N acetate buffer (pH 5.5). Typically, 100  $\mu\text{l}$  ethanol and ca. 0.1 mg of the bis(thiosemicarbazone) ligand dissolved in 1–2  $\mu\text{l}$  DMSO was added to the aqueous  $^{67}\text{Cu}$ -acetate (30–100  $\mu\text{l}$ ). The resulting solution was diluted with saline to ca. 5% ethanol and  $\leq 0.5\%$

DMSO (final  $^{67}\text{Cu}$  concentration of  $\sim 2 \mu\text{Ci}/\mu\text{l}$ ) and then filtered through a 0.2- $\mu\text{m}$  PTFE membrane before use. The radiochemical purity of the  $^{67}\text{Cu}$ -bis(thiosemicarbazone) complexes was determined by thin-layer chromatography on silica gel plates eluted with ethanol and was always found to exceed 99%. The  $^{67}\text{Cu}$ -PTSM,  $^{67}\text{Cu}$ -PTS,  $^{67}\text{Cu}$ -ETS,  $^{67}\text{Cu}$ -*n*-PrTS,  $^{67}\text{Cu}$ -*n*-BuTS,  $^{67}\text{Cu}$ -KTS and  $^{67}\text{Cu}$ -ETSM complexes migrate with  $R_f$  values of 0.78, 0.89, 0.91, 0.82, 0.89, 0.94 and 0.87, respectively, while the  $^{67}\text{Cu}$ -acetate synthetic precursor remains at the origin ( $R_f = 0.0$ ).

### Ultrafiltration Studies with $^{67}\text{Cu}$ Complexes

Plasma protein binding of the  $^{67}\text{Cu}$ -bis(thiosemicarbazone) complexes was quantitatively evaluated by ultrafiltration. Each Amicon Centrifree® (Beverly, MA) ultrafiltration device (30,000 Dalton NMWL) was loaded with 300–600  $\mu\text{l}$  of either plasma, albumin solution (35 mg/ml saline) or normal saline (control) within 2 min of mixing with the  $^{67}\text{Cu}$ -complex. Typically, 1 ml protein solution was mixed with 1–2  $\mu\text{l}$   $^{67}\text{Cu}$ -bis(thiosemicarbazone) solution. The Centrifree® devices were loaded and immediately ( $< 1$  min) centrifuged in a Sorvall RC2-B Refrigerated Centrifuge (20°C) with a SS-34 45° fixed angle rotor at  $1000 \times g$  for 20 min. Copper-67-bis(thiosemicarbazone) complex concentrations (cpm/ml) in the unfiltered protein solutions and their ultrafiltrates were determined by counting measured aliquots in a Packard Autogamma 5530 automatic gamma counter (Downer's Grove, IL). The percentage of free (unbound)  $^{67}\text{Cu}$ -bis(thiosemicarbazone) complex was calculated as:

$$\left[ \frac{(^{67}\text{Cu-L concentration in protein ultrafiltrate})}{(^{67}\text{Cu-L concentration in unfiltered protein solution})} \right] \cdot \left[ \frac{(^{67}\text{Cu-L concentration in saline ultrafiltrate})}{(^{67}\text{Cu-L concentration in unfiltered saline solution})} \right]^{-1} \cdot 100\%.$$

### RESULTS

Consistent with earlier findings of interspecies variability in the plasma/RBC and albumin/RBC partitioning of  $^{67}\text{Cu}$ -PTSM (10), ultrafiltration studies of tracer binding to plasma proteins and serum albumin reveal that  $^{67}\text{Cu}$ -PTSM is bound much more strongly by HSA than by DSA (Fig. 2). Approximately 62% of the lipophilic  $^{67}\text{Cu}$ -PTSM tracer is protein-bound in both dog plasma and in saline solutions of DSA that approximate the albumin concentration of plasma. This is in marked contrast to the behavior of  $^{67}\text{Cu}$ -PTSM in human plasma or saline solutions of HSA, where 95%–97% of the tracer appears to be protein-bound.

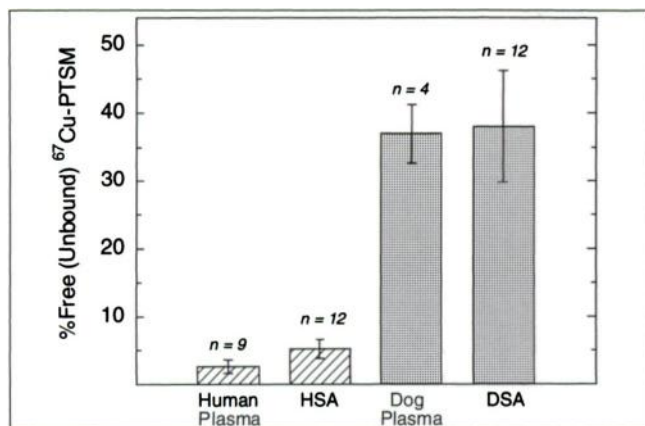
In rat biodistribution studies, a number of bis(thiosemicarbazone) complexes related to Cu-PTSM (Table 1) have been shown to exhibit myocardial uptake and retention similar to that observed with  $^{67}\text{Cu}$ -labeled Cu-PTSM (15). To determine whether interspecies variability in albumin binding is a general feature of complexes of this type, the plasma/RBC partitioning of  $^{67}\text{Cu}$ -PTSM and six related tracers was evaluated in both dog and human blood (Fig. 3). Since slow reductive decomposition of these tracers is known to occur in blood (10), the plasma/RBC partitioning measurements were made 1 min postmixing at both 4°C and 37°C. In no case did the data obtained at 37°C

**TABLE 1**  
Copper(II) Bis(thiosemicarbazone) Complexes Studied\*

Complex (Cu-L)	$R_1$	$R_2$	Log $P^{\dagger}$
$^{67}\text{Cu}$ -PTSM	$-\text{CH}_3$	$-\text{CH}_3$	$1.92 \pm 0.04$
$^{67}\text{Cu}$ -PTS	$-\text{CH}_3$	$-\text{H}$	$0.76 \pm 0.04$
$^{67}\text{Cu}$ -ETS	$-\text{CH}_2\text{CH}_3$	$-\text{H}$	$1.35 \pm 0.02$
$^{67}\text{Cu}$ - <i>n</i> -PrTS	$-\text{CH}_2\text{CH}_2\text{CH}_3$	$-\text{H}$	$1.78 \pm 0.05$
$^{67}\text{Cu}$ - <i>n</i> -BuTS	$-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$	$-\text{H}$	$2.30 \pm 0.03$
$^{67}\text{Cu}$ -KTS	$-\text{CH}(\text{CH}_3)\text{OCH}_2\text{CH}_3$	$-\text{H}$	$1.71 \pm 0.04$
$^{67}\text{Cu}$ -ETSM	$-\text{CH}_2\text{CH}_3$	$-\text{CH}_3$	$2.65 \pm 0.07$

\*Functional substituents ( $R_1$  and  $R_2$ ) are numbered as shown in Figure 1.

$^{\dagger}$ Octanol/water partition coefficient,  $P$ , from Ref. 15.



**FIGURE 2.** Binding of <sup>67</sup>Cu-PTSM to plasma proteins and serum albumin as measured by ultrafiltration. Values shown represent the mean and s.d. of *n* measurements. All values have been corrected for nonspecific binding of the lipophilic tracer to the ultrafiltration device using binding data independently measured for <sup>67</sup>Cu-PTSM in protein-free normal saline solution (*n* = 22).

differ substantially from that obtained at 4°C, indicating that the tracers remained intact under both conditions for the brief time frame of the experiment. Therefore, data from both 4°C and 37°C has been combined in the results presented in Figure 3.

In dog blood, all of the <sup>67</sup>Cu-*bis*(thiosemicarbazone) tracers were found to exhibit similar plasma/RBC partitioning with ~75% of the tracer found in the RBC phase and ~25% in the plasma phase (Fig. 3). However, in human blood the plasma/RBC partitioning of these tracers is more complex. When compared with the results from dog blood, the <sup>67</sup>Cu-PTS, <sup>67</sup>Cu-KTS, <sup>67</sup>Cu-*n*-BuTS and <sup>67</sup>Cu-ETSM complexes, like <sup>67</sup>Cu-PTSM, are found to exhibit substan-

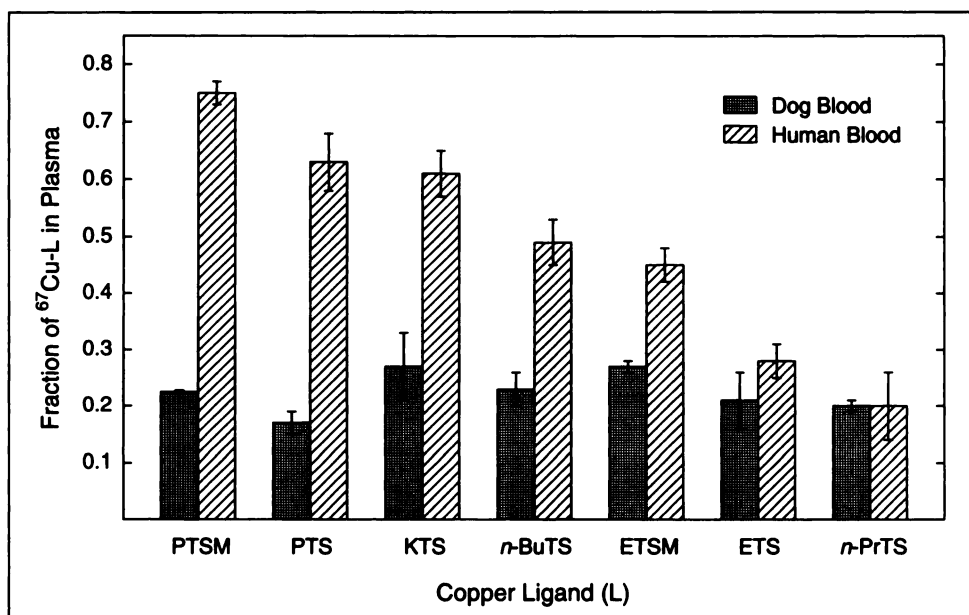
**TABLE 2**  
Binding of <sup>67</sup>Cu(II)-*Bis*(thiosemicarbazone) Complexes to Serum Albumin: Examination of Species-Dependence by Ultrafiltration\*

Complex (Cu-L)	Free (unbound) <sup>67</sup> Cu-L determined by ultrafiltration (%)	
	Dog albumin (35 mg/ml saline)	Human albumin (35 mg/ml saline)
<sup>67</sup> Cu-PTSM	40.2 ± 5.2	5.0 ± 1.0
<sup>67</sup> Cu-ETS	44.3 ± 4.6	41.5 ± 3.0
<sup>67</sup> Cu- <i>n</i> -PrTS	30.3 ± 1.7	37.0 ± 2.0

\*Values shown represent the mean ± s.d. of 6–12 measurements. All values are corrected for nonspecific binding of tracer to the ultrafiltration device using binding data independently measured for tracer in protein-free saline solution.

tially greater tracer partitioning into the plasma phase of human blood. This contrasts with the behavior of <sup>67</sup>Cu-ETS and <sup>67</sup>Cu-*n*-PrTS, in which the plasma/RBC partitioning of tracer in human blood exhibits virtually no difference from the partitioning of these two tracers (and Cu-PTSM) in dog blood.

To more precisely assess the strength of the association of Cu-ETS and Cu-*n*-PrTS with serum albumin, ultrafiltration studies were performed to measure the binding of <sup>67</sup>Cu-ETS and <sup>67</sup>Cu-*n*-PrTS to HSA and DSA (Table 2). The results obtained in concurrent measurements using <sup>67</sup>Cu-PTSM (Table 2) are consistent with those from the earlier independent study (Fig. 2) and confirm the reproducibility of both the measurement and the interspecies variability in Cu-PTSM binding to serum albumin. As suggested by the plasma/RBC partitioning results (Fig. 3), the



**FIGURE 3.** Partitioning of <sup>67</sup>Cu-*bis*(thiosemicarbazone) complexes between the plasma and RBC fractions of whole blood (in vitro). Values represent the mean and s.d. of four measurements (human blood hematocrit = 44%; dog blood hematocrit = 56%).

**TABLE 3**  
Biodistribution of Copper-67-PTSM in Rats: Effect of Premixing with Albumin

	%ID organ (2 min postinjection)		
	Cu-PTSM	Cu-PTSM + HSA	Cu-PTSM + DSA
Blood	6.53 ± 0.25	6.27 ± 0.34	6.67 ± 0.19
Heart	2.65 ± 0.40	2.38 ± 0.08	2.31 ± 0.12
Lungs	5.01 ± 0.54	4.85 ± 0.73	5.09 ± 0.51
Liver	11.2 ± 1.9	13.6 ± 3.77	11.6 ± 0.6
Kidney (1)	2.87 ± 0.10	2.99 ± 0.56	2.77 ± 0.18
Brain	2.87 ± 0.48	2.64 ± 0.32	2.59 ± 0.35
n	4	3	4
Rat mass	192–206 g	174–182 g	167–190 g

binding of Cu-ETS and Cu-*n*-PrTS to human serum albumin (Table 2) appears to be no stronger than the binding of these two tracers (or Cu-PTSM) to dog serum albumin.

Substantial nonspecific tracer association with the ultrafiltration membrane was observed in control ultrafiltration measurements. Ultrafiltration of protein-free saline solutions of the <sup>67</sup>Cu-*bis*(thiosemicarbazone) tracers reveals significant, but reproducible, depletion of tracer in the ultrafiltrate, despite the obvious necessity that 100% of tracer is “free” (i.e., the extent of protein binding must be zero). Apparent % free <sup>67</sup>Cu-L values from these control ultrafiltration measurements with tracer in normal saline were: 61.4 ± 2.7, 71.5 ± 4.7 and 77.3 ± 2.5 for <sup>67</sup>Cu-PTSM, <sup>67</sup>Cu-ETS and <sup>67</sup>Cu-*n*-PrTS, respectively [(cpm in ultrafiltrate)/(cpm in unfiltered saline solution)<sup>-1</sup> · 100%]. The protein binding results reported in Figure 2 and Table 2 are corrected for this nonspecific binding to the ultrafiltration device.

A number of other commercial ultrafiltration systems were also briefly examined for these measurements and similarly found to exhibit significant nonspecific tracer association with the membrane. The disposable devices employed in the reported studies were selected for their convenience and reproducibility. Nonspecific radiotracer association with the ultrafiltration membrane did not appear to vary as a function of ultrafiltrate volume.

Previous rat biodistribution experiments have shown that premixing <sup>67</sup>Cu-PTSM with rat plasma prior to intravenous administration does not alter tracer biodistribution (10). To assess the reversibility of tracer Cu-PTSM binding to HSA, the biodistribution of <sup>67</sup>Cu-PTSM was evaluated in rats following intravenous injection of tracer in ~0.2 ml of either 5% ethanol:95% saline solution or 35 mg/ml solutions of HSA or dog albumin. The presence of dog or human albumin in the injectate produced no effect on <sup>67</sup>Cu-PTSM biodistribution (Table 3).

## DISCUSSION

It is an accepted pharmacological principle that only the nonprotein-bound or free fraction of a drug in blood is

available for distribution to tissue (16–18). For the majority of drugs, albumin accounts for almost the entire drug binding observed in plasma (17). In its role as a drug carrier, the binding capacity of albumin is generally considered to be nonsaturable (16–20). Since a drug bound to albumin is practically nondiffusible (21), strong albumin binding of a radiopharmaceutical will directly impair its utility as a perfusion tracer.

The available methods for measurement of drug binding to plasma proteins have been critically reviewed (22). These techniques include ultrafiltration studies to directly quantitate albumin binding, as well as simple measurements of the plasma/erythrocyte partitioning of drug in whole blood (10,23,24). In the present work with a number of [<sup>67</sup>Cu]-copper(II) *bis*(thiosemicarbazone) complexes, measurements of plasma/RBC partitioning have been used for screening to assess the relative strength of tracer binding to DSA and HSA. These partitioning studies were followed by ultrafiltration measurements with selected compounds for more detailed evaluation of the relative strengths of tracer-albumin interactions. The ultrafiltration technique separates the aqueous phase (and “free” drug) from protein (and protein-bound drug) at the ultrafiltration membrane (22), providing results that reflect the magnitude of the equilibrium constant, *K* (Equation 2).

Detailed studies of the interspecies variability in partitioning of <sup>67</sup>Cu-PTSM between plasma and red cells (10), as well as ultrafiltration studies of the binding of <sup>67</sup>Cu-PTSM to albumin (Fig. 2), show that human albumin binds Cu-PTSM much more strongly than does dog albumin (i.e., *K*<sub>human albumin</sub> > *K*<sub>dog albumin</sub>; Equations 1 and 2). Although it is not uncommon to find some interspecies variability in the binding of drugs to albumin, the relatively large disparity between DSA and HSA binding observed with Cu-PTSM is unusual (24–28). We believe the observed (9,11–14) attenuation of <sup>62</sup>Cu-PTSM myocardial uptake in humans at high rates of myocardial blood flow indicates that *k*<sub>2</sub> (Equation 1) has become the rate-limiting step for tissue extraction of tracer. (At resting flows the myocardial uptake of <sup>62</sup>Cu-PTSM in humans is adequately predicted by dog imaging data, while at high flows in humans the capillary transit time apparently becomes sufficiently rapid that albumin-binding affects the myocardial extraction of tracer.)

It is clear from this and earlier studies (10) that the Cu-PTSM molecule remains intact in its interaction with albumin and that this interaction is reversible. Solvent extraction and TLC studies demonstrate that the tracer is chemically stable in the presence of plasma (10). Furthermore, <sup>67</sup>Cu-PTSM premixed with rat plasma (10), or premixed with dog or human albumin (Table 3), exhibits the same biodistribution in rats as <sup>67</sup>Cu-PTSM in saline.

We have previously identified several copper(II) *bis*(thiosemicarbazone) derivatives related to Cu-PTSM that also exhibit the desired myocardial uptake and retention of the Cu radiolabel following intravenous injection (Cu-PTS, Cu-ETS, Cu-KTS, Cu-*n*-PrTS, Cu-*n*-BuTS, and Cu-ETSM)

(15). In light of the interspecies variability of albumin binding observed with Cu-PTSM, we have reinvestigated these other  $^{67}\text{Cu}$ -bis(thiosemicarbazone) complexes to determine whether species-dependent albumin binding is a general feature of compounds of this type, or whether one of these other compounds would be more promising than  $^{62}\text{Cu}$ -PTSM for investigation as a perfusion tracer in humans. From studying the albumin binding of these  $^{67}\text{Cu}$ -radiotracers (Fig. 3 and Table 2), we can identify two distinct classes of Cu-bis(thiosemicarbazone) complexes:

1. Cu(II)-bis(thiosemicarbazone) complexes that interact more strongly with HSA than dog albumin (Cu-PTSM, Cu-PTS, Cu-*n*-BuTS, Cu-KTS, Cu-ETSM); and
2. Cu(II)-bis(thiosemicarbazone) complexes that exhibit no preferential association with HSA over dog albumin (Cu-ETS, Cu-*n*-PrTS).

While Cu-ETS and Cu-*n*-PrTS clearly interact less strongly with HSA than do the other copper(II)-bis(thiosemicarbazone) complexes examined, the underlying chemical basis for this observation remains unclear. Neither Cu-ETS nor Cu-*n*-PrTS present any obviously unique structural features and their octanol/water partition coefficients (Table 1) are comparable to the compounds where substantial variation in albumin binding is observed between species.

## CONCLUSION

Copper-PTSM has been shown to exhibit significant interspecies variability in the strength of its binding to serum albumin, interacting much more strongly with HSA than with DSA. This finding appears to explain problems that have been encountered in using animal data to predict the behavior of  $^{62}\text{Cu}$ -PTSM as a myocardial perfusion tracer in humans. Most of the related Cu(II)-bis(thiosemicarbazone) complexes examined were found to exhibit interspecies variability of albumin binding similar to that observed with Cu-PTSM. However, two complexes were identified that exhibit no preferential association with HSA. We are currently evaluating the complexes in this second category,  $^{62}\text{Cu}$ -ETS and  $^{62}\text{Cu}$ -*n*-PrTS, to determine their utility as tracers for measurement of regional tissue perfusion.

## ACKNOWLEDGMENTS

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