

# Optimization of labeling and metabolite analysis of copper-64-labeled azamacrocyclic chelators by radio-LC-MS

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## Abstract

The cross-bridged tetraamine ligand 4,11-bis(carboxymethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane (H<sub>2</sub>CB-TE2A) allows formation of a radio-copper complex with higher in vivo stability than that of the corresponding non-cross-bridged analog 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA). The structure of the <sup>nat</sup>Cu(II) complex of CB-TE2A has been previously determined by X-ray crystallography; however, direct high-pressure liquid chromatography (HPLC) characterization of the corresponding <sup>64</sup>Cu complex was inaccessible due to the inability to detect the complex by ultraviolet absorbance at the radiotracer level. A reverse-phase HPLC separation of a series of <sup>nat</sup>Cu(II)-tetraazamacrocyclic complexes, both traditional and cross-bridged, was developed and applied toward characterization and assessment of the purity of the corresponding no-carrier-added <sup>64</sup>Cu-labeled complexes. Verification of the identity of copper-64-labeled compounds was also achieved by coupling this HPLC method with mass spectrometry. The radio-liquid chromatography/mass spectrometry methodology was further extended to study the in vivo metabolic fates of <sup>64</sup>Cu-azamacrocyclic complexes.

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**Keywords:** Copper-64; Azamacrocyclic; HPLC; Radio-LC-MS; Impurity identification; Metabolite analysis

## 1. Introduction

Copper-64 is a useful diagnostic and therapeutic radio-nuclide in nuclear medicine due to its half-life ( $t_{1/2} = 12.7$  h), decay characteristics ( $\beta^+$ , 7.4%;  $\beta^-$ , 39%) and the capability for large-scale production with high specific activity on a biomedical cyclotron [1,2]. Increased use of <sup>64</sup>Cu and other

Cu radioisotopes in both positron emission tomography and targeted radiotherapy applications has created a need for copper chelators with high in vivo stability. The development of optimal chelators for Cu(II) is of considerable importance when designing systems for the in vivo delivery of copper radioisotopes.

Polyaminopolycarboxylate macrocyclic ligands are commonly used for complexation of metals for radiopharmaceutical applications. However, the Cu(II) complexes of these ligands are susceptible to metal dissociation in vivo, releasing copper ions that readily bind proteins. The commercially available macrocyclic ligands 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (H<sub>4</sub>TETA) and 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (H<sub>4</sub>DOTA) (Fig. 1) have been extensively used for chelation of copper radionuclides in clinical imaging and therapy studies involving both antibodies and peptides [3–9]. However, the in vivo dissociation and subsequent binding of radiometals to proteins have been demonstrated in normal rats [10,11]. The macrocyclic ligands 4,11-bis(carboxy-

*Abbreviations:* HPLC, high-pressure liquid chromatography; PDA, photodiode array; UV, ultraviolet;  $\lambda_{\max}$ , wavelength of maximal absorbance; RP, reverse phase; LC-MS, liquid chromatography/mass spectrometry; ESI, electrospray ionization; ES<sup>+</sup>, electrospray (positive ionization mode); PET, positron emission tomography; H<sub>2</sub>CB-TE2A, 4,11-bis(carboxymethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane; H<sub>4</sub>TETA, 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid; H<sub>2</sub>CB-DO2A, 4,10-bis(carboxymethyl)-1,4,7,10-tetraazabicyclo[5.5.2]tetradecane; H<sub>4</sub>DOTA, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; H<sub>3</sub>TE3A, 1,4,8,11-tetraazacyclotetradecane-1,4,8-triacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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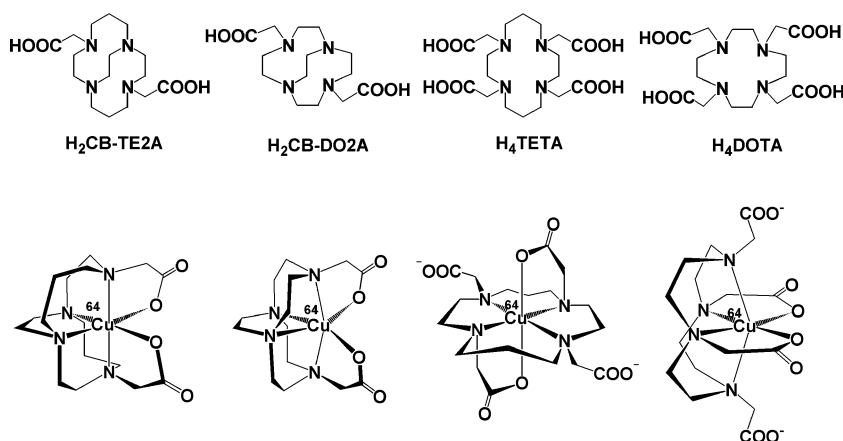


Fig. 1. Structural comparison of  $\text{H}_2\text{CB-TE2A}$ ,  $\text{H}_2\text{CB-DO2A}$ ,  $\text{H}_4\text{TETA}$  and  $\text{H}_4\text{DOTA}$  (top), and structural representations of the corresponding  $^{64}\text{Cu}$ -labeled complexes based on solved crystal structures (bottom).

methyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane ( $\text{H}_2\text{CB-TE2A}$ ) and 4,10-bis(carboxymethyl)-1,4,7,10-tetraazabicyclo[5.5.2]tetradecane ( $\text{H}_2\text{CB-DO2A}$ ) [12] are analogs of  $\text{H}_4\text{TETA}$  and  $\text{H}_4\text{DOTA}$ , respectively, where two of the acetate arms present have been replaced by ethylene bridges between nonadjacent nitrogens (Fig. 1). These ligands have been shown to form  $\text{Cu(II)}$  complexes [12,13] with superior kinetic stability and improved biological behavior compared to their nonbridged analogs [11,14].

Development of a chromatographic separation of these azamacrocyclic complexes would allow for the determination of radiochemical purity and would also provide a means for the detection of metabolites from biological samples. Accomplishing these goals would facilitate the evaluation of azamacrocyclic complexes as carriers of copper radionuclides in radiopharmaceutical applications. In studies reported previously, an ion-exchange chromatographic technique was developed for studying the lability of  $^{64}\text{Cu}$  complexes with ethylenediaminetetraacetic acid (EDTA) and other acyclic chelators in an aqueous system [15]. A reverse-phase (RP) separation of a radiolabeled  $^{153}\text{Gd}$  azamacrocyclic complex was developed using a C-18 column and a buffered aqueous eluant [16]. Herein we report the development of a RP method for the separation of  $^{64}\text{Cu}$ -labeled azamacrocyclic complexes.

Mass spectrometry coupled to high-pressure liquid chromatography (HPLC) (LC-MS) has proven to be a valuable tool for the analysis of compounds being developed as radiopharmaceuticals, allowing for detection of trace-level impurities [17], determination of specific activity [18] and identification of metabolites [19–21]. An LC-MS methodology for separation of  $^{64}\text{Cu}$ -labeled azamacrocyclic complexes would be advantageous because of the requirement of high sensitivity for detection and characterization of radiometal complexes at or near the tracer level. Incorporation of  $^{64}\text{Cu}$  into the desired ligand may be confirmed by comparing the retention times of radiolabeled species with the appropriate characterized  $^{\text{nat}}\text{Cu}$  complexes detected by LC-MS. This radio-LC-MS approach was used to confirm

the identities of  $^{99\text{m}}\text{Tc}$  Sestamibi [22] and other  $^{99\text{m}}\text{Tc}$  radiopharmaceuticals [23,24], a process that is usually achieved at the tracer level only indirectly by assessment of RP-HPLC retention times. The experiments described herein were directed toward confirmation of the formation of desired  $^{64}\text{Cu}$ -azamacrocyclic complexes at the tracer level, identification of  $^{64}\text{Cu}$ -labeled impurities and investigation of the extent of  $^{64}\text{Cu}$ -azamacrocyclic complex metabolism in vivo.

## 2. Materials and methods

### 2.1. General procedures and materials

Cold copper salts and solvents were commercial grade. Cross-bridged ligands  $\text{H}_2\text{CB-TE2A}$  and  $\text{H}_2\text{CB-DO2A}$  were prepared as trifluoroacetate salts by modifications of published methods [12,13].  $\text{H}_4\text{TETA}$  obtained from Aldrich Co. (Milwaukee, WI) was used to collect the data shown in Fig. 7;  $\text{H}_4\text{TETA}$  obtained from Macrocylics (Dallas, TX) was used to collect the data shown in Figs. 2, 8 and 9;  $\text{H}_4\text{DOTA}$  was obtained from Macrocylics.

$^{\text{nat}}\text{Cu(II)} \cdot \text{TETA} \cdot 2\text{H}_2\text{O}$  [25],  $^{\text{nat}}\text{Cu(II)} \cdot \text{DOTA}$  [25],  $[\text{natCu(II)} \cdot \text{CB-TE2A} \cdot \text{Na}(\text{H}_2\text{O})\text{ClO}_4]_2 \cdot \text{H}_2\text{O}$  [13] and  $^{\text{nat}}\text{Cu(II)} \cdot \text{CB-DO2A}$  [11] were prepared according to published methods. Samples for injection were prepared by dissolving the crystals in mobile phase prior to injection; typical injection volumes were 50 or 100  $\mu\text{l}$ . A solution containing all four  $^{\text{nat}}\text{Cu}$  complexes, each at a concentration of 1.25 mg/ml, was analyzed to obtain the data for Fig. 2. Analysis of a 5 mg/ml solution of crude  $^{\text{nat}}\text{Cu-CB-TE2A}$  gave the data shown in Fig. 3. Analysis of a 5-mg/ml solution of recrystallized  $^{\text{nat}}\text{Cu-TETA}$  provided the data shown in Fig. 7.

### 2.2. Radiochemistry

Copper-64 was prepared on the Washington University Medical School CS-15 cyclotron by the  $^{64}\text{Ni}(p,n)^{64}\text{Cu}$  nuclear reaction at a specific activity range of 50–200 mCi/ $\mu\text{g}$  as previously described [1]. No-carrier-added  $^{64}\text{Cu}$ -

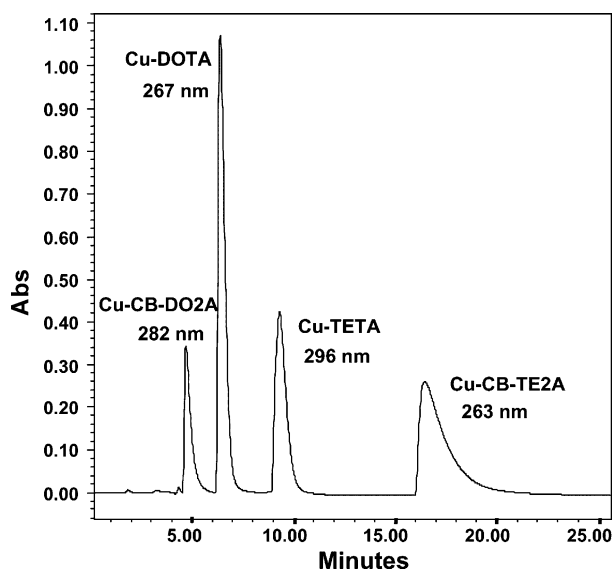


Fig. 2. Ultraviolet elution profile (0.5 ml/min) of recrystallized  $^{nat}\text{Cu}$ -tetraazamacrocyclic complexes (1:1 molar ratios) in 0.1% formic acid/ $\text{H}_2\text{O}$  on a C8 column.

TETA was prepared by addition of a solution of  $^{64}\text{CuCl}_2$  in 0.1–0.5 M HCl to a 5-mM solution of the appropriate ligand in 0.1 M ammonium citrate buffer (pH 7.5) at 25 °C.

The initial method for preparing the  $^{64}\text{Cu}$ -CB-TE2A complex involved incubation of a 200- $\mu\text{l}$  aliquot of  $\text{H}_2\text{CB-TE2A}$  (5 mM) in aqueous 0.1 M ammonium citrate buffer (pH 7.5) with 1 mCi of  $^{64}\text{Cu}$  at 75 °C for 3–4 h.

Due to the formation of radiolabeled impurities (see Results), the radiolabeling method was modified to the following procedure. A  $\text{Cs}_2\text{CO}_3$ -saturated solution of a 200- $\mu\text{l}$  aliquot of  $\text{H}_2\text{CB-TE2A}$  in ethanol (5 mM) was refluxed for 30 min,  $^{64}\text{CuCl}_2$  was added, and the reaction mixture was refluxed for an additional 30-min period to achieve formation of  $^{64}\text{Cu}$ -CB-TE2A with concomitant precipitation of insoluble CsCl. The resulting supernatant was easily isolated following centrifugation and was subsequently transferred to aqueous citrate buffer by allowing the ethanol to evaporate during an additional incubation period.

### 2.3. Radio-LC-MS

High-pressure liquid chromatography analyses were performed using an Agilent Zorbax StableBond SB-C8, 3.5  $\mu\text{m}$ , 75 $\times$ 4.6 mm analytical column (Agilent Technologies, Palo Alto, CA) with an isocratic mobile phase consisting of  $\text{H}_2\text{O}/0.1\%$  formic acid (pH 2.5) at a flow rate of 0.25 or 0.50 ml/min. A double end-capped Agilent Zorbax XDB-C8 column was also tested in an attempt to alleviate the peak tailing and drifting but provided no retention of any of the complexes under identical conditions. A Waters 600E system controller was operated using Waters Millennium software. The complexes were monitored using a Waters 996 photodiode array (PDA) detector (220–310 nm range), a radioactive detector and a Waters ZQ 4000 single quadrupole mass spectrometer equipped with an electrospray ionization (ESI) LC-MS interface. The ESI-MS was

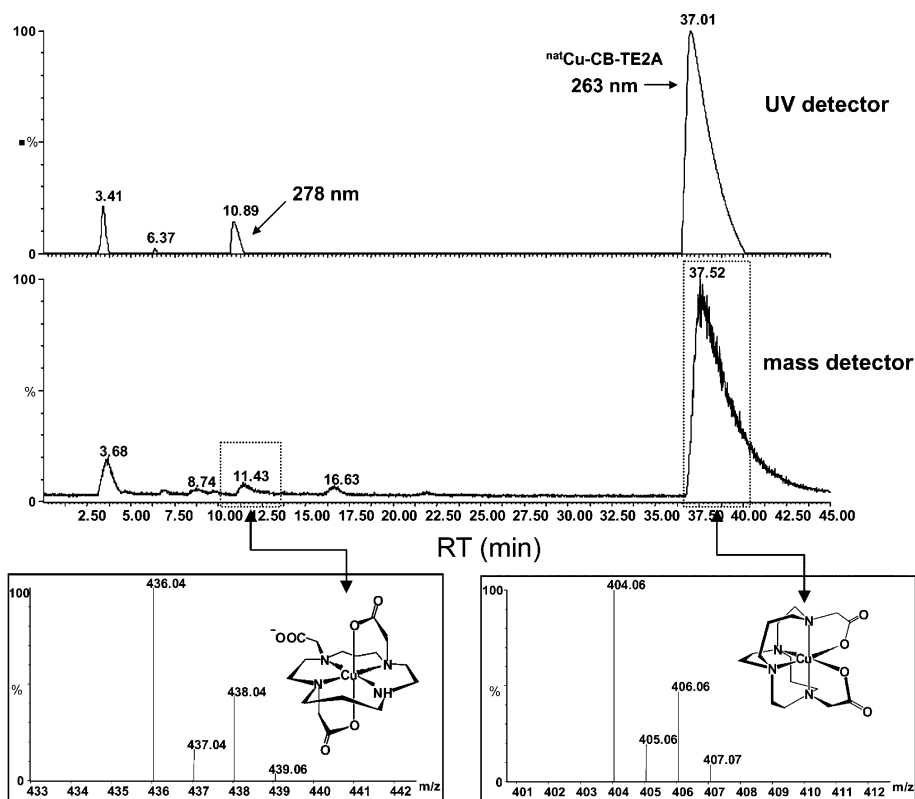


Fig. 3. LC-MS chromatogram (0.25 ml/min) revealing a 436-Da impurity ( $^{nat}\text{Cu}$ -TE3A) in crude  $^{nat}\text{Cu}$ -CB-TE2A derived from unpurified ligand.

operated in the positive-ion mode under the following conditions: capillary voltage, 3.0 kV; cone voltage of 55 V; desolvation temperature, 300 °C; source temperature, 110 °C; cone gas (N<sub>2</sub>) flow rate, 65 L/h; desolvation gas (N<sub>2</sub>) flow rate, 260 L/h. Selected ion recording analysis was used to detect ions for quantification along with a full mass spectra scan in the range of *m/z* 130–800 to detect decomposition products or impurities. All radiolabeled complexes were diluted with mobile phase prior to injection.

#### 2.4. Metabolism studies

All animal studies were performed in accordance with procedures approved by the Washington University Animal Studies Committee. The metabolic fates of carrier-added <sup>64</sup>Cu-CB-TE2A, <sup>64</sup>Cu-TETA, <sup>64</sup>Cu-CB-DO2A, and <sup>64</sup>Cu-DOTA in the liver and blood of male Lewis rats were analyzed as previously described [11]. Highly concentrated (~5 mg/ml) carrier-added samples (100 µl) for tail-vein injection were prepared via addition of each <sup>nat</sup>Cu-azamacrocyclic complex to a 10- to 15-mCi quantity of the corresponding no-carrier-added Cu-64-labeled complex. The rats were sacrificed at 4 h postinjection, and the livers were immediately excised and placed on ice. Tissue samples were homogenized in 65:35 ethanol/ammonium acetate buffer (0.1 M, pH 5.5) using a Tissumizer tissue homogenizer (Tekmar, Cincinnati, OH) followed by a 1-min tip sonication using a Sonifier 185 cell disruptor (Branson, Danbury, CT). The insoluble protein and cellular debris were removed by centrifugation at 23,500 × *g* for 30 min at 4 °C. The resulting cleared lysates from liver extraction were passed through Centricon-10 concentrators in order to isolate them from soluble proteins (Millipore, Billerica, MA) and subjected to LC-MS.

To ensure that low-molecular-weight species were not trapped in passage through the centricon membrane, a control experiment was performed in which the cleared lysates from liver extraction were analyzed by size-exclusion HPLC on a Superose 12 HR 10/30 column (Amersham Biosciences, Uppsala, Sweden) equilibrated with 20 mM HEPES, 150 mM NaCl (pH 7.3). The resulting low-molecular-weight fraction (void volume) was concentrated under reduced pressure and also subjected to LC-MS. Comparable results were obtained using either method.

### 3. Results and discussion

#### 3.1. Reverse-phase chromatography

The elution profile of each of the series of four <sup>nat</sup>Cu(II) azamacrocyclic complexes on a RP C-8 column (non-end-capped) equilibrated with aqueous formic acid (0.1%, pH 2.5, 0.5 ml/min) was assessed by monitoring the ultraviolet (UV) absorbance maximum as a function of elution time using a PDA detector operating between 220 and 310 nm, with each complex possessing a unique absorption maximum and retention time as indicated in Fig. 2. The retention

time of Cu(II)-CB-TE2A was found to vary with the concentration of complex injected, with less concentrated samples having longer retention times. Specifically, retention times as long as 25 min were observed for no-carrier-added <sup>64</sup>Cu-CB-TE2A despite the 16- to 17-min retention time observed for carrier-added (1:1) <sup>64</sup>Cu-CB-TE2A and for the corresponding <sup>nat</sup>Cu complex. Although the cause of these fluctuations is not fully understood, it is believed to involve interactions of this neutral complex with uncapped silanol binding sites on the stationary phase, with certain sites possibly becoming saturated upon injection of increasing concentrations of Cu(II)-CB-TE2A. A comparable end-capped C8 column was also tested in an attempt to alleviate the peak tailing and drifting, but it provided no retention for any of the complexes. However, this phenomenon of concentration-dependent variations in retention time was predictable and reproducible, and the identity of the Cu(II)-CB-TE2A complex in both the early- and late-eluting trials was verified by MS. A similar behavior was observed for Cu(II)-CB-DO2A, although the elution times varied over a much narrower range.

#### 3.2. LC-MS analysis

Upon radiolabeling under no-carrier-added radiotracer conditions (aqueous ammonium citrate buffer, pH 7.5), the retention time of the <sup>64</sup>Cu-labeled species detected in the radiometric channel was inconsistent with that of <sup>nat</sup>Cu-CB-TE2A, suggesting that an impurity was radiolabeled preferentially over CB-TE2A. Upon sequential addition of (1) <sup>64</sup>Cu and (2) <sup>nat</sup>Cu at a 1:10 Cu/ligand molar ratio, complete incorporation of <sup>64</sup>Cu into trace ligand impurities was observed (see Supporting Information). The retention time of the <sup>64</sup>Cu-labeled trace impurity at 0.5 ml/min was 7 min compared to 21 min for the <sup>nat</sup>Cu-CB-TE2A complex. In light of these results, mass spectrometry was employed to characterize the ligand in the absence of Cu(II), revealing only the expected mass ion for H<sub>2</sub>CB-TE2A (see Supporting Information). This prompted an LC-MS analysis of the <sup>nat</sup>Cu complex of CB-TE2A at the macroscopic level in an attempt to detect the impurity. It has been shown that reducing the ESI flow rate leads to increased desolvation, ionization, and ion-transfer efficiency over ESI conducted at higher flow rates, all contributing to significant improvements in concentration and mass sensitivity at the lower flow rates [26,27]. As a result, a flow rate of 0.25 ml/min rather than 0.5 ml/min was selected in an attempt to achieve higher sensitivity, as the purity of the ligand in the absence of copper suggested that the impurity was present in very low amounts. Initially, the mass spectrometer parameters were adjusted to maximize sensitivity of the different labeled complexes being examined. However, it was found that this was not required due to the similar nature of these compounds and that one set of parameters could be used. Consequently, mass chromatograms obtained from several different runs could be directly compared. LC-MS analysis of a crude solid <sup>nat</sup>Cu-CB-TE2A sample allowed the UV

(RT=10.9 min) and mass (RT=11.4 min) detection of a  $^{nat}\text{Cu}$ -associated impurity having the characteristic natural isotopic distribution pattern expected for Cu ( $^{63}\text{Cu}$  69.17%,  $^{65}\text{Cu}$  30.83%) (Fig. 3). It is of paramount importance to note, however, that the analyte under scrutiny in this case was crude Cu(II)-CB-TE2A complex prepared from unpurified ligand derived from unpurified direct precursors to allow easy detection of impurity complex(es).

The observed mass peak eluting at 11.4 min corresponded to a molecular weight of 436 Da, consistent with Cu(II)-1,4,8,11-tetraazacyclotetradecane-1,4,8-triacetic acid [Cu(II)-TE3A]. The isotopic distribution pattern obtained also matched the theoretical pattern calculated for Cu(II)-TE3A. The formation of  $\text{H}_3\text{TE3A}$  as a trace byproduct in the synthesis of  $\text{H}_2\text{CB-TE2A}$  cannot be absolutely ruled out [12]. However, as previously pointed out,  $\text{H}_3\text{TE3A}$  was not detected in LC-MS investigations of free  $\text{H}_2\text{CB-TE2A}$ . Alternatively,  $\text{H}_3\text{TE3A}$  could arise via oxidation of  $\text{H}_2\text{CB-TE2A}$  in solution in the presence of Cu(II), but we have no independent proof of such a mechanism.

A void volume peak (3.4 min) and an additional UV peak (6.4 min) were also observed; however, the isotopic distribution patterns indicated that no Cu-containing species eluted in either of these peaks. In order to positively identify  $\text{H}_3\text{TE3A}$  as the impurity, an authentic standard was synthesized according to literature procedures [28]. The resulting Cu(II)-TE3A complex eluted with a retention time of 11.5 min, with the expected mass of 436 Da (see Supporting Information). As anticipated, the elution time, mass spectral data, and isotopic splitting patterns were consistent with that of the impurity shown in Fig. 3.

An isotopic dilution study was performed by labeling CB-TE2A with  $^{64}\text{Cu}$  in the presence of various amounts of  $^{nat}\text{Cu}$  in an attempt to assess the relative amounts of trace impurities present in  $\text{H}_2\text{CB-TE2A}$  (Fig. 4). It is important to note, however, that the amounts and types of trace impurities apparently vary by batch of ligand, and the amounts of Cu-labeled impurities vary with labeling conditions. Radiolabeling CB-TE2A in citrate buffer at a  $^{nat}\text{Cu}$ /ligand ratio of 1:100 resulted in a mixture of  $^{64}\text{Cu}$ -CB-TE2A and multiple  $^{64}\text{Cu}$ -labeled impurities (Fig. 4A). Two of the  $^{nat}\text{Cu}$ -labeled impurities (377 and 365 Da) were unexpectedly accompanied by later eluting mass peaks for the corresponding  $^{nat}\text{Zn}$  complexes (Fig. 4B and C), while a third impurity of 219 Da did not have an associated  $^{nat}\text{Zn}$  peak (Fig. 4D). These results confirmed the simultaneous presence of both  $^{64}\text{Cu}$ -CB-TE2A and radiolabeled impurities. Furthermore, the addition of  $^{nat}\text{Cu}$  at a  $^{nat}\text{Cu}$ /ligand ratio of 1:100 was able to partially block incorporation of  $^{64}\text{Cu}$  into these impurities, suggesting that the impurities were present in relatively low amounts. This is consistent with the mass spectrum (see Supporting Information) and nuclear magnetic resonance spectrum of the free ligand, neither of which indicated the presence of impurities.

Development of an improved radiolabeling procedure was deemed the most practical solution to the undesirable

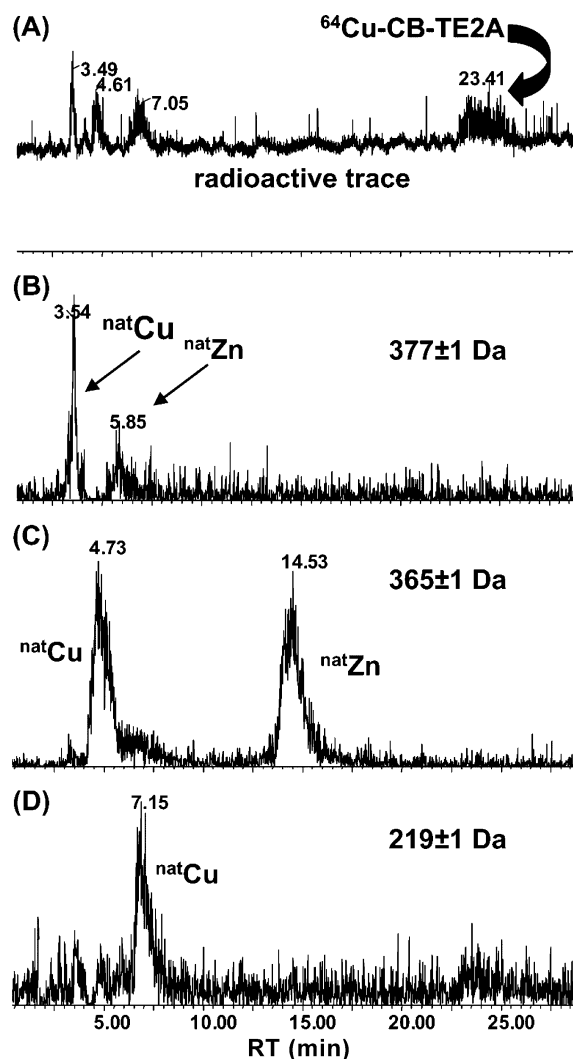


Fig. 4. Radio-LC-MS (0.5 ml/min) revealing multiple  $^{64}\text{Cu}$ -labeled impurities in carrier-added (1:100)  $^{64}\text{Cu}$ -CB-TE2A (citrate labeling method): (A) radioactive trace and corresponding single mass ion elution profiles for (B)  $377\pm 1$  Da, (C)  $365\pm 1$  Da and (D)  $219\pm 1$  Da.

complexation of  $^{64}\text{Cu}$  by  $\text{H}_3\text{TE3A}$  and other trace impurities. We employed a new method of radiolabeling designed to circumvent complexation of  $^{64}\text{Cu}$  by kinetically favored impurities. Two strategies were used to accomplish this goal: (1) performing the metal complexation at an elevated temperature to drive the formation of the thermodynamic product and (2) performing the complexation under basic conditions to increase the concentration of CB-TE2A relative to  $\text{H}_2\text{CB-TE2A}$ . Sequential treatment of  $\text{H}_2\text{CB-TE2A}$  with excess  $\text{Cs}_2\text{CO}_3$  and  $^{64}\text{CuCl}_2$  in refluxing ethanol satisfied both of these requirements and resulted in formation of radiochemically pure  $^{64}\text{Cu}$ -CB-TE2A, even under no-carrier-added conditions. Comparison of the radiolabeled species formed at  $25^\circ\text{C}$  (Fig. 5A) versus reflux ( $78^\circ\text{C}$ ) (Fig. 5B) illustrates the temperature dependence of this kinetically disfavored metal complexation.

Radiolabeling by refluxing in ethanol with excess  $\text{Cs}_2\text{CO}_3$  at a  $^{nat}\text{Cu}$ /ligand molar ratio of 1:100 resulted in

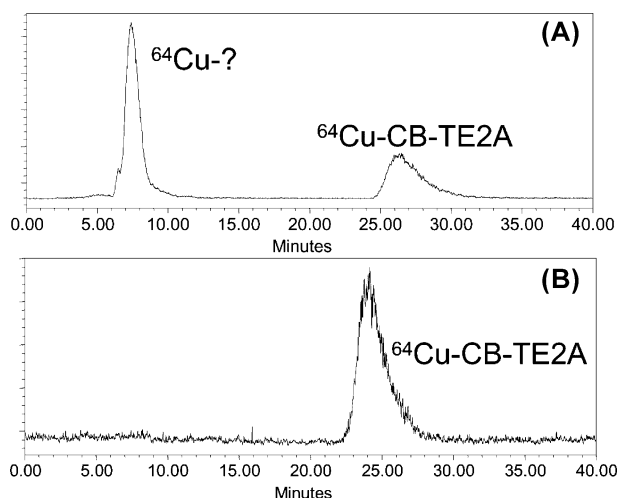


Fig. 5. Radio-HPLC chromatograms (0.5 ml/min) of no-carrier-added  $^{64}\text{Cu}$ -CB-TE2A following incubation in ethanol with excess  $\text{Cs}_2\text{CO}_3$  at (A) 25 °C and (B) 78 °C.

the formation of radiochemically pure  $^{64}\text{Cu}$ -CB-TE2A (RT=21.9 min) having the expected UV ( $\lambda_{\text{max}}=263$  nm) and mass (404.1 Da) peaks (Fig. 6). A mass peak for  $^{\text{nat}}\text{Zn}$ -CB-TE2A (405.1 Da, RT=28.7 min) was also observed. The detectable levels of  $^{\text{nat}}\text{Zn}$  complexation by CB-TE2A and by other ligands was unexpected; possible sources of this metal include plastic containers created using zinc molds, reagents used for ligand syntheses, buffer salts, or the milli-Q water system. Precedence exists for the formation of Zn-tetraazamacrocyclic complexes, as characterizations of Zn-TETA [25], Zn-DOTA [25] and Zn-CB-DO2A [29] have been reported. To test the hypothesis that the detected impurity was caused by Zn chelation,  $^{\text{nat}}\text{Zn}$ -CB-TE2A was prepared independently from zinc sulfate and  $\text{H}_2\text{CB-TE2A}$  under the same conditions used to form the

analogous copper complex. The resulting complex gave a retention time (~27 min), mass (405 Da) and isotopic distribution pattern that were all consistent with the data in Fig. 6 and matching the theoretical isotopic distribution pattern for  $^{\text{nat}}\text{Zn}$ -CB-TE2A (see Supporting Information). This finding also demonstrates a powerful advantage of LC-MS, as the presence of these UV-inactive, nonradioactive Zn complexes would have otherwise gone undetected.

The presence of trace impurities was not limited to the cross-bridged ligand  $\text{H}_2\text{CB-TE2A}$ . An impurity in the commercially available chelator  $\text{H}_4\text{TETA}$  was revealed by the presence of UV (RT=4.5 min) and mass (RT=4.9 min) peaks of an additional  $^{\text{nat}}\text{Cu}$  complex in a recrystallized sample of  $^{\text{nat}}\text{Cu-TETA}$  (Fig. 7). Interestingly, both the UV absorption maximum (278 nm) and the MS-determined molecular weight (436 Da) of this  $^{\text{nat}}\text{Cu(II)}$ -complexed impurity were identical to that of  $\text{Cu-TE3A}$ , the same impurity observed for  $\text{H}_2\text{CB-TE2A}$ . It is plausible that this impurity originates from incomplete N-functionalization during the commercial synthesis of  $\text{H}_4\text{TETA}$  from cyclam.

### 3.3. Metabolite analysis

The metabolic fates of the  $^{64}\text{Cu}$ -labeled azamacrocycles were investigated by analysis of samples extracted following in vivo metabolism in rats. This was accomplished by analysis of liver extracts obtained from rats 4 h following intravenous injection of highly concentrated (5 mg/ml) carrier-added  $^{64}\text{Cu}$ -CB-TE2A or  $^{64}\text{Cu-TETA}$ . The liver extracts were passed through Centricon-10 filtration membranes to remove high-molecular-weight (>10,000 Da) proteins, and the resulting low-molecular-weight eluents were analyzed by radio-HPLC and then compared to radio-HPLC traces of purified  $^{64}\text{Cu-TETA}$  and  $^{64}\text{Cu-CB-TE2A}$

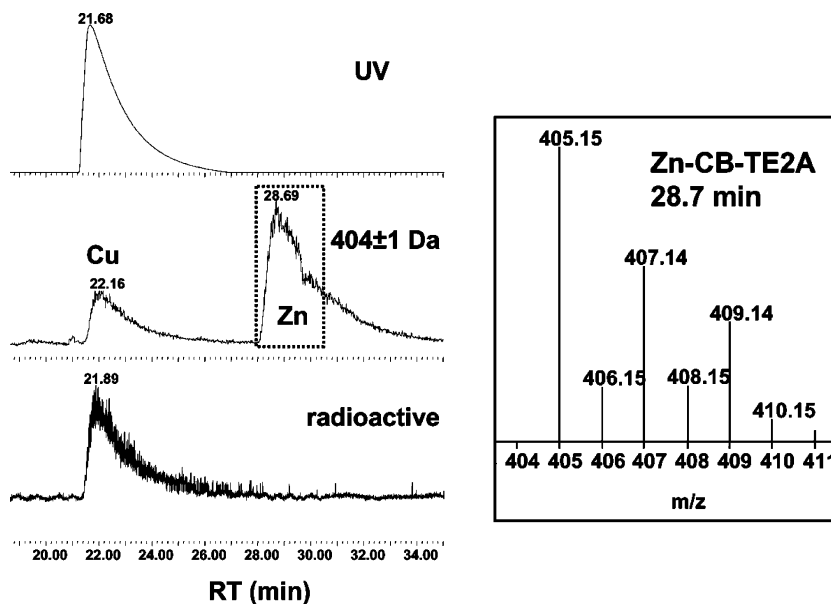


Fig. 6. Radio-LC-MS concordance (0.5 ml/min) of carrier-added (1:100)  $^{64}\text{Cu}$ -CB-TE2A ( $\text{Cs}_2\text{CO}_3$ /ethanol labeling method) and mass spectral splitting pattern of  $^{\text{nat}}\text{Zn}$ -CB-TE2A (inset).

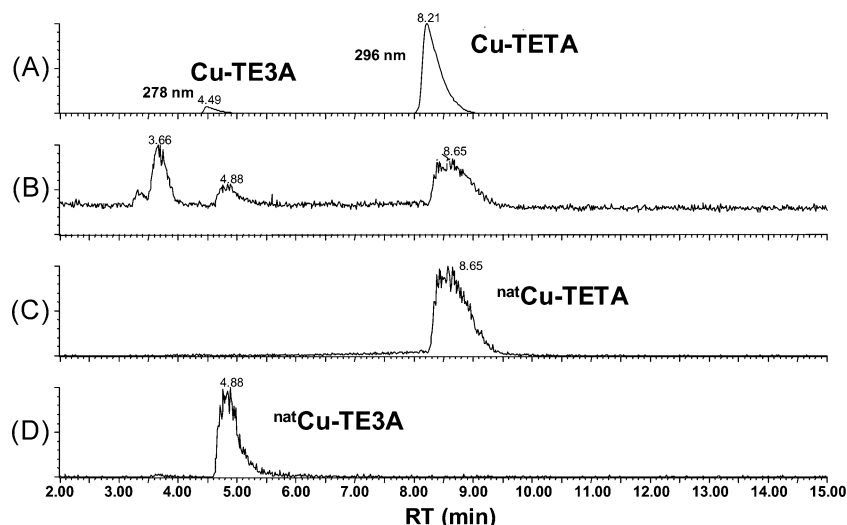


Fig. 7. LC-MS chromatogram (0.5 ml/min) of recrystallized  $^{nat}\text{Cu}$ -TETA formed from commercially obtained  $\text{H}_4\text{TETA}$ : (A) UV elution profile, (B) total mass elution profile and extracted single mass ion elution profiles corresponding to (C) Cu-TETA (494 Da) and (D) Cu-TE3A (436 Da).

(Fig. 8A and B, respectively). The resulting radio-LC chromatograms indicated that  $^{64}\text{Cu}$ -TETA was metabolized to a significant extent in rat liver (Fig. 8C), in contrast to  $^{64}\text{Cu}$ -CB-TE2A, which remained predominantly intact (Fig. 8D).

The retention time of the metabolite sample containing  $^{64}\text{Cu}$ -CB-TE2A (Fig. 8D) did not correlate with the retention time of the  $^{64}\text{Cu}$ -CB-TE2A prior to injection into the rat (Fig. 8B) due to concentration-dependent shifts in retention time. As a result, LC-MS was used to confirm that intact  $^{64}\text{Cu}$ -CB-TE2A was the species responsible for both peaks. Many components in biological fluids such as ionic salts and bile acids can reduce the analyte response through ionization suppression to such an extent that major metabolites are rendered undetectable by MS [30]. As a result, a flow rate of 0.25 ml/min was selected in an attempt to achieve higher sensitivity. A portion of the rat liver extract eluant obtained at 4 h was analyzed by LC-MS and the mass elution data (Fig. 9A) examined for any copper-containing species. The mass spectrum of a late eluting peak (~46 min) revealed the

presence of the intact Cu-CB-TE2A complex at (Fig. 9B), while no other Cu-containing species could be detected. A similar analysis was performed on liver extracts obtained at 4 h for rats receiving carrier-added (1:1)  $^{64}\text{Cu}$ -TETA. A thorough search of the total mass elution data (Fig. 9C) revealed no detectable quantities of intact Cu-TETA (MW=494 Da), suggesting that significant metabolism had occurred. Furthermore, the very low levels of radioactivity recovered from the liver extracts following injection of  $^{64}\text{Cu}$ -TETA compared to that obtained with  $^{64}\text{Cu}$ -CB-TE2A suggest that very little low-molecular-weight-bound Cu is present in rat liver at 4 h postinjection. This could be partially due to transchelation of Cu into proteins, conversion to another low-molecular-weight metabolite by a hepatic enzyme, or both. Supporting the latter is the observation of what appears to be a single detectable Cu-containing species eluting at 13.8 min (Fig. 9D) with a mass of 578 Da (bottom inset). This molecular weight (Cu-TETA+84) is consistent with a derivative of Cu-TETA having undergone

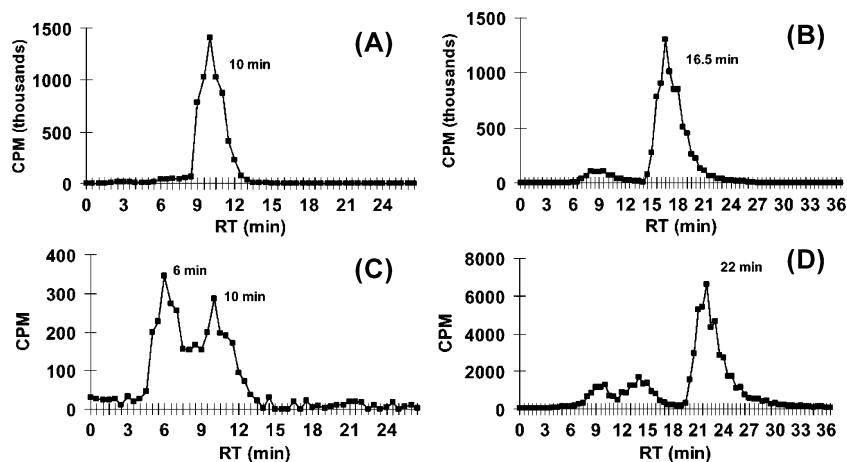


Fig. 8. Reverse-phase HPLC (0.5 ml/min) resolution of carrier-added (A)  $^{64}\text{Cu}$ -TETA and (B)  $^{64}\text{Cu}$ -CB-TE2A injectates and the corresponding rat liver metabolites of carrier-added (C)  $^{64}\text{Cu}$ -TETA and (D)  $^{64}\text{Cu}$ -CB-TE2A at 4 h postinjection.

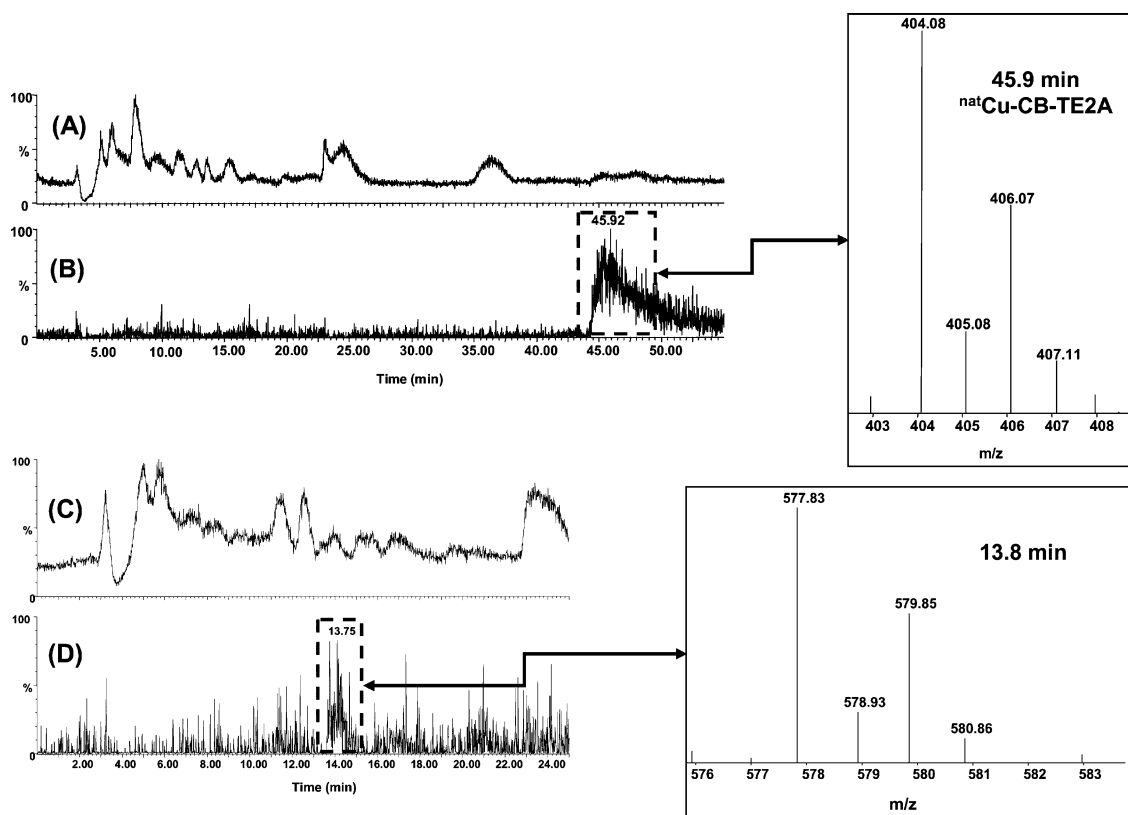


Fig. 9. LC-MS chromatograms (0.25 ml/min) of rat liver extracts (4 h postinjection) following intravenous injection of carrier-added (A) Cu-CB-TE2A (total mass elution), (B) Cu-CB-TE2A (single mass ion elution profile of 404 Da), (C) Cu-TETA (total mass elution) and (D) Cu-TETA (single mass ion elution profile of 578 Da). The splitting patterns of intact Cu-CB-TE2A (top inset) and a putative Cu-containing metabolite (bottom inset) is also shown.

propyl esterification at the two noncoordinating carboxylates, although this seems unlikely. Moreover, the low concentrations of this putative Cu-complex results in a high signal-to-noise ratio of the mass spectral data making it difficult to draw any definitive conclusions from these data. Nevertheless, it is quite evident that after 4 h *in vivo* there is very little, if any, intact Cu(II)-TETA present in the liver.

#### 4. Conclusions

The presence of trace impurities in synthetic ligands such as H<sub>2</sub>CB-TE2A is unavoidable. However, reactions performed at radiotracer levels often require higher standards of purity. Comparative analysis of the <sup>nat</sup>Cu- versus <sup>64</sup>Cu-labeled species by radio-LC-MS revealed kinetically favored complexation of <sup>64</sup>Cu by trace impurities at the no-carrier-added level. Positive identification of a Cu-complexed impurity was achieved, and an alternative radiolabeling strategy was developed to favor the formation of the <sup>64</sup>Cu-CB-TE2A complex. The unanticipated incorporation of <sup>64</sup>Cu into a trace chelator impurity illustrates the importance of characterizing radiolabeled complexes by comparison to known standards using reliable chromatographic methods. Our results illustrate that radio-LC-MS is a powerful technique that can facilitate the analysis of radiolabeled compounds to be evaluated as radiopharmaceuticals by verification of their

identities, by assessment of their radiochemical purities and by evaluation of the radiolabeled species present in biological samples following *in vivo* metabolism.

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## Supporting Information

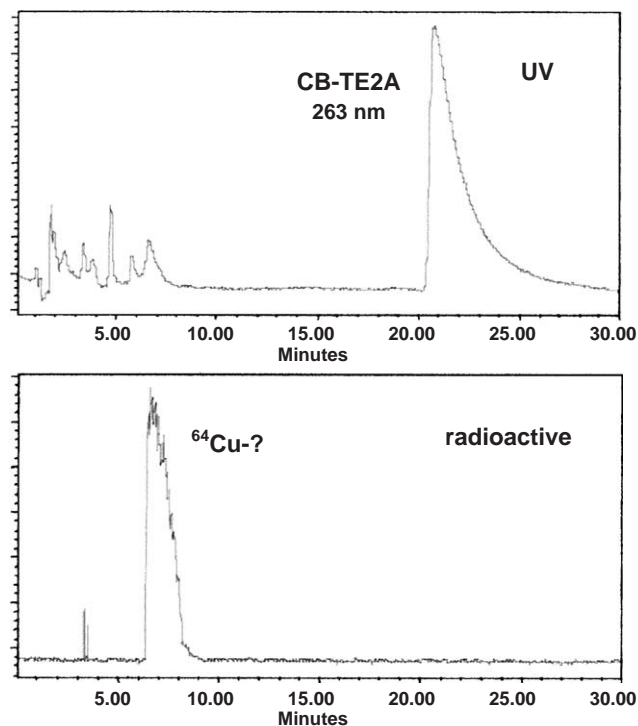
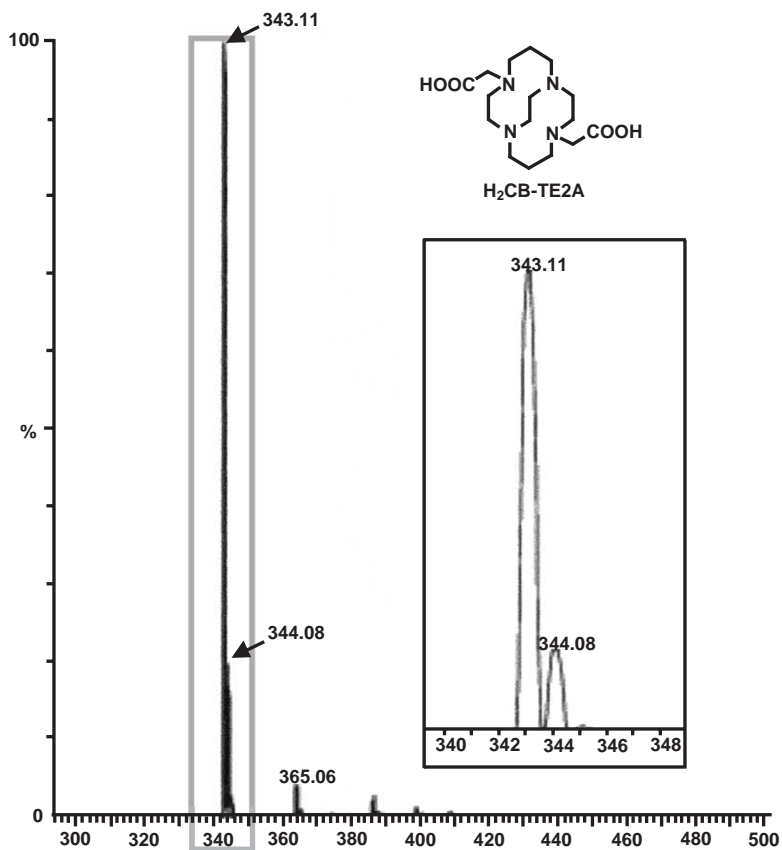
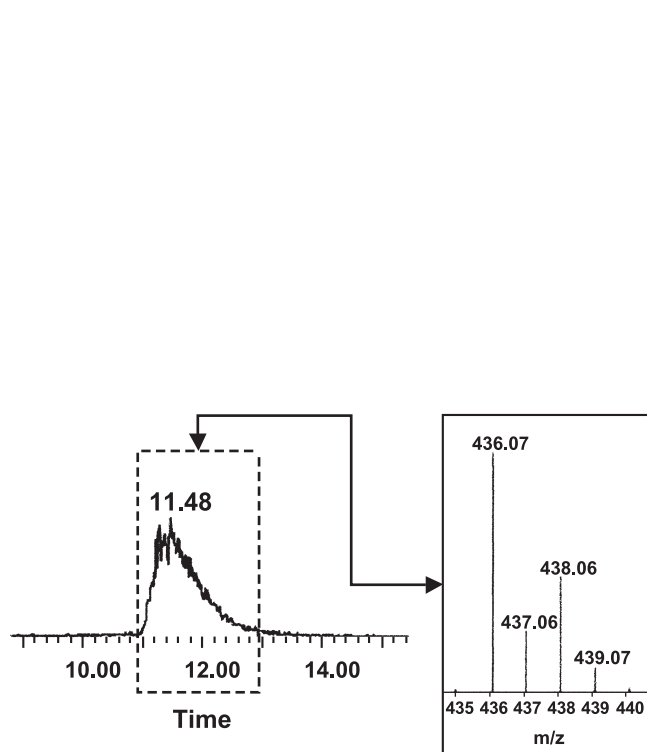
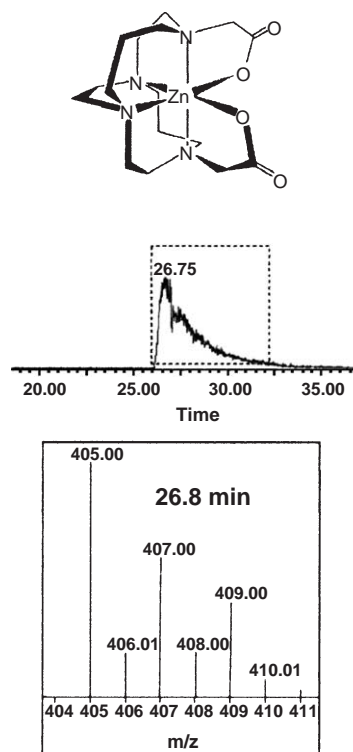


Fig. S1. HPLC resolution (0.5 mL/min) of carrier-added (1:10 molar ratio)  $^{64}\text{Cu}$ -CB-TE2A ( $^{64}\text{Cu}$  added prior to  $^{nat}\text{Cu}$ ) revealing the inconsistency between UV-detected  $^{nat}\text{Cu}$ (II)-CB-TE2A (top) and a  $^{64}\text{Cu}$ -labeled trace impurity (bottom).

Fig. S2. Mass spectrum of  $H_2CB-TE2A$  in the absence of copper.Fig. S3. LC-MS (0.25 mL/min) of a synthesized authentic standard of  $Cu(II)-TE3A$ .Fig. S4. LC-MS (0.5 mL/min) of a synthesized authentic standard of  $^{nat}Zn-CB-TE3A$ .