

Investigation of copper–azamacrocyclic complexes by high-performance liquid chromatography

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ABSTRACT: The use of copper radioisotopes in imaging and therapy has prompted an increased interest in chelators which form stable copper complexes, such as Cu(II)–azamacrocyclic complexes. The effects of charge, stability and the size of the macrocyclic backbone of the Cu(II)–azamacrocyclic complexes on biological behavior have been evaluated. Here we report a reversed-phase high-performance liquid chromatography (HPLC) method to separate several Cu(II)–azamacrocyclic complexes, including Cu(II) complexes of 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (TETA), 4,11-bis(carboxymethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane (CB-TE2A) and 4,10-bis(carboxymethyl)-1,4,7,10-tetraazabicyclo[5.5.2]tetradecane (CB-DO2A). Absorbance at 280 nm was used to monitor the complexes as they eluted from the reversed-phase column. The effects of the concentration of the buffer, the pH of the buffered mobile phase and the concentration of the organic modifier, methanol, on the separation were investigated. Separation of these copper complexes by ion-pair HPLC with the use of a mass spectrometry-compatible ion-pair reagent, triethylammonium acetate, in the mobile phase at pH 6.3 is also presented. The reversed-phase chromatographic conditions utilized also allow the pK_a s of Cu–TETA and the $\log(k'_w)$ values of Cu–CB-TE2A, Cu–TETA and Cu–CB-DO2A to be estimated. Copyright © 2005 John Wiley & Sons, Ltd.

KEYWORDS: Cu–azamacrocyclic complexes; reversed-phase HPLC; ion-pair HPLC; dissociation constant; pK_a ; lipophilicity; $\log(k'_w)$

INTRODUCTION

Copper radionuclides offer a wide range of half-lives and several decay profiles, providing a useful selection of both diagnostic and therapeutic isotopes (Blower *et al.*, 1996; Anderson and Welch, 1999; Reichert *et al.*, 1999). Among the available copper radionuclides, ⁶⁴Cu has favorable properties for use in both positron emission tomography (PET) imaging and targeted radiotherapy. Among these properties are its half-life, decay scheme and the possibility of large-scale production with high specific activity using a biomedical cyclotron (Blower *et al.*, 1996; Connet *et al.*, 1996; McCarthy *et al.*, 1997; Anderson and Welch, 1999; Reichert *et al.*, 1999). The increasing use of ⁶⁴Cu and other copper

radioisotopes in nuclear medicine has led to a need for the development of bifunctional chelators (BFCs). A BFC consists of a chelator which complexes the radiometal and a functional group for attaching the chelated metal to a biomolecule. The BFC must possess high *in vivo* stability against the loss of copper radioisotope during *in vivo* delivery (Moi *et al.*, 1985; Rogers *et al.*, 1996; Jones-Wilson *et al.*, 1998; Sun *et al.*, 2002). Synthesis of several novel cross-bridged tetraamine ligands having non-adjacent nitrogens connected by an ethylene (CH₂CH₂) bridge and their Cu(II) complexes have been reported (Weisman *et al.*, 1990, 1996; Wong *et al.*, 2000; Niu *et al.*, 2004). The *in vivo* stabilities of some ⁶⁴Cu-labeled conventional and cross-bridged tetraazamacrocyclic complexes have been studied and compared (Anderson *et al.*, 1995; Rogers *et al.*, 1996; Jones-Wilson *et al.*, 1998; Sun *et al.*, 2002; Sprague *et al.*, 2004; Boswell *et al.*, 2004). It has been shown that the charge and the size of the macrocyclic backbone of the complexes have significant effects on the *in vivo* stability of the copper complexes. The development of means for separating and characterizing these complexes is critical for assessing their properties and monitoring their *in vivo* behavior.

Reversed-phase high-performance liquid chromatography (RP-HPLC) has emerged as the most popular HPLC separation technique due to its selectivity and

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Abbreviations used: BFCs, bifunctional chelators; CB-DO2A, 4,10-bis(carboxymethyl)-1,4,7,10-tetraazabicyclo[5.5.2]tetradecane; CB-TE2A, 4,11-bis(carboxymethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane; PET, positron emission tomography; TEAA, triethylammonium acetate; TETA, 1,4,8,11-tetraazacyclotetradecane-1,4,8,11-tetraacetic acid.

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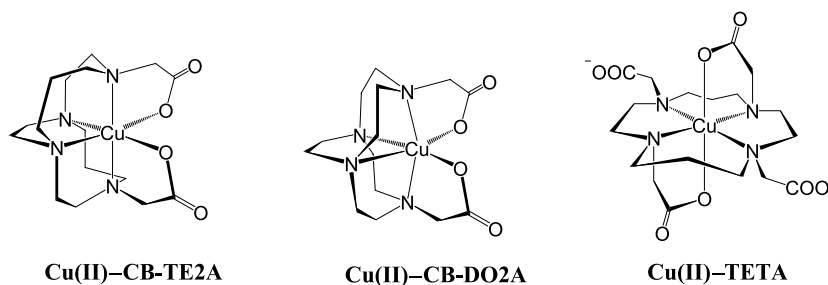


Figure 1. Structures of Cu–CB–TE2A, Cu–CB–DO2A and Cu–TETA.

versatility. It can be applied to a great variety of samples, such as polar compounds, including many pharmaceuticals. Ionic compounds can be separated by means of secondary equilibria such as ion-pair (Hearn, 1985) and ligand exchange (Cagniant, 1992). RP-HPLC has also been employed for the assessment of physico-chemical properties of solutes (Hafkenschied and Tomlinson, 1986; Li and Cai, 2001), including the acid dissociation constant (pK_a ; Horváth *et al.*, 1977; Hardcastle and Jano, 1998; Manderscheid and Eichinger, 2003), complex formation constant (Horváth *et al.*, 1979; Takayanagi *et al.*, 2001), and the lipophilicity or hydrophobicity parameter (Braumann, 1986; Minick *et al.*, 1988; Lambert, 1993; Dorsey and Khaledi, 1993; Poole and Poole, 2003). The need for only small amounts of impure sample represents a major advantage of measuring such properties using RP-HPLC methods.

Separation of copper complexes from other metal complexes with the same type of ligand by RP-HPLC has been reported (Timerbaev *et al.*, 1991; Wang and Lee, 1997; Giovannetti and Bartocci, 1998). RP-HPLC separation of copper complexes having different types of ligands have also been reported, such as the separations of copper(II)-chelated bleomycin congeners (Klett *et al.*, 1984) and copper complexes of chlorophylls and their derivatives (Inoue *et al.*, 1994, 1996). The copper-64-labeled azamacrocyclic complexes discussed herein have been analyzed by LC-MS (Boswell *et al.*, 2005). The separation of Cu–azamacrocyclic complexes was achieved by the use of an octyl-bonded phase (C_8) column and a mobile phase containing 0.1% aqueous formic acid at pH 2.5. Problematic peak tailing and variable retention times of one of the Cu–azamacrocyclic complexes were observed (Boswell *et al.*, 2005).

Here we report conditions which provide improved RP-HPLC separations of three Cu–azamacrocyclic complexes. Two of these complexes are uncharged in aqueous solution, Cu(II)–4,11-bis(carboxymethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane (Cu–CB–TE2A) and Cu(II)–4,10-bis(carboxymethyl)-1,4,7,10-tetraazabicyclo[5.5.2]tetradecane (Cu–CB–DO2A), while the third, Cu(II)–1,4,8,11-

tetraazacyclotetradecane-1,4,8,11-tetraacetic acid (Cu–TETA), is anionic at neutral pH. The structures of these complexes are presented in Fig. 1. Absorbance at 280 nm was used to monitor the complexes as they eluted from the reversed-phase column. Separation was achieved with the use of two different mobile phases, a citric acid-buffered mobile phase and a formic acid mobile phase, and two different columns, C_{18} and C_8 . The effects of the pH of the buffered mobile phase, the concentration of the buffer, and the concentration of the organic modifier, methanol, on the separation were studied. Separation based on ion-pair liquid chromatography was also investigated with the use of MS-compatible, triethylammonium acetate (TEAA) as the ion-pair reagent. Estimations of the pK_a s of Cu–TETA and the lipophilicity of the complexes based on RP-HPLC data are also discussed.

EXPERIMENTAL

Reagents. Certified anhydrous citric acid was purchased from Fisher (Fairlawn, NJ, USA). Reagent-grade formic acid solution (88% w/w) was purchased from J. T. Baker (Phillipsburg, NJ, USA). Glacial acetic acid (99.7%) was purchased from VWR Scientific (San Francisco, CA, USA). Triethylamine (99%) was purchased from Lancaster Synthesis (Windham, NH, USA). HPLC-grade methanol was purchased from Pharmco (Brookfield, CT, USA). A 1.0 M stock solution of the ion-pair reagent, TEAA, was prepared by titrating aqueous triethylamine with acetic acid to a pH of 6.3 (Huber and Krajete, 1999). Certified sodium hydroxide solution (50% w/w) and reagent-grade hydrochloric acid (36.5% w/w) were purchased from Fisher (Fairlawn, NJ, USA). Dilute solutions of these reagents were used to adjust the pH of the mobile phases. The deionized water used in the preparation of the standard solutions and eluents was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA). All mobile phases were filtered through a 0.45 μ m nylon filter (Whatman, Hillboro, OR, USA) prior to use.

Apparatus. Chromatographic separations were performed at ambient temperature using a Nicolet LC 9560 HPLC system (Madison, WI, USA) fitted with a Reodyne 7125 injector (Cotati, CA, USA) having a 10 μ L injection loop. A Kratos Spectroflow 783 UV–vis absorbance detector with the wave-

length set at 280 nm was used for chromatographic detection in combination with a Kipp & Zonen BD41 chart recorder. Two reversed-phase HPLC analytical columns were used for the chromatographic separation: (1) a Betabasic C₁₈ column (150 × 4.6 mm, 5 μm; Keystone Scientific, Bellefonte, PA, USA); and (2) a Zorbax SB-C₈ column (75 × 4.6 mm, 3.5 μm; Agilent, Wilmington, DE, USA).

Chromatographic conditions. The reversed-phase HPLC column was washed for at least 30 min using 60:40 (v/v) methanol:water at 1.0 mL/min after daily use. The chromatographic column was equilibrated with the desired mobile phase for at least 30 min before use. For ion-pair HPLC separations, the column was equilibrated with the mobile phase containing the ion-pair reagent for at least 60 min. A mobile phase flow rate of 1.0 mL/min was used throughout the study. The pH of 0.1% formic acid solution (approximately equivalent to 22 mM formic acid) was 2.5.

The dead time (t_0) used for the calculation of the capacity factor, $k' = (t_R - t_0)/t_0$, where t_R is the retention time of analyte, was measured as the time of the first distortion of the baseline after the injection of water (Rimmer *et al.*, 2002).

Sample preparation. Cu(II)–TETA (Riesen *et al.*, 1986), Cu(II)–CB–TE2A (Wong *et al.*, 2000) and Cu(II)–CB–DO2A (Boswell *et al.*, 2004) were prepared according to the published methods. Another Cu(II)–CB–TE2A sample [called crude Cu(II)–CB–TE2A] was recovered from Cu(II)–CB–TE2A samples which were used to study acid decomplexation in aqueous hydrochloric acid solution. These Cu(II)–CB–TE2A samples were evaporated to dryness, washed with ether, and re-dissolved in 95% ethanol. After centrifugation to remove the small amount of precipitate, the clear blue solutions were placed in ether chambers to precipitate the crude Cu(II)–CB–TE2A. All samples of the complexes for analysis by HPLC were dissolved in deionized water.

Data analysis. The statistical software JMP (SAS Institute Inc., Cary, NC, USA) was employed to perform nonlinear fitting to estimate the pK_a s of Cu(II)–TETA.

RESULTS AND DISCUSSION

Effect of the concentration of the citric acid buffer on the capacity factors

The separation of four Cu(II)–azamacrocyclic complexes on a C₈ reversed-phase column was previously studied with a mobile phase of 22 mM aqueous formic acid solution at pH 2.5 (Boswell *et al.*, 2005). However, severe peak tailing and inconsistent retention times of one of the Cu–azamacrocyclic complexes, Cu–CB–TE2A, were encountered. A buffer may be added to the mobile phase to control pH and affect selectivity, and with these chromatographic conditions reproducible separations may be achieved with acceptable peak shape. Citric acid has three pK_a values, 3.128, 4.761 and 6.396 (Martell and Smith, 1974), thereby providing a

wide range of useful buffering regions ($pK_a \pm 1$ pH unit) including the region of pH 2.5. For these reasons, citrate was chosen as a mobile phase buffer for the separation of these Cu–azamacrocyclic complexes. The effect of the concentration of citric acid buffer at pH 2.5 from 12 to 150 mM on the capacity factors of the Cu–azamacrocyclic complexes was evaluated with the use of a C₁₈ column. As the concentration of the citric acid buffer increased, the capacity factors of the Cu–azamacrocyclic complexes decreased slightly. The decreased capacity factors of the complexes at citric acid buffer concentrations over 60 mM led to a reduction in the resolution of Cu–TETA and Cu–CB–DO2A and the co-elution of an impurity with Cu–CB–DO2A. Figure 2 shows representative chromatograms at three concentrations of the citric acid in the mobile phase, 30, 60 and 150 mM.

Effect of the pH of the mobile phase on the capacity factors

Of the three Cu–azamacrocyclic complexes, two complexes, Cu–CB–TE2A and Cu–CB–DO2A, are expected to be uncharged in aqueous solution. The third complex, Cu–TETA, is ionizable in aqueous solution over a wide pH range due to its having two extra carboxylic acid functionalities (Fig. 1). The retention of the ionizable Cu–TETA is expected, therefore, to be highly dependent on the pH of the mobile phase. The effect of the pH of the mobile phase on the retention of these three complexes on a C₁₈ column is presented in Fig. 3. As the pH of the mobile phase increased from 2.2 to 4.5, the capacity factor of Cu–TETA decreased, while the capacity factors of Cu–CB–TE2A and Cu–CB–DO2A increased only slightly. Increasing the pH promotes the ionization of Cu–TETA. This results in a decrease in the retention of Cu–TETA because the ionic form of Cu–TETA, a negatively charged species, is more hydrophilic than the neutral form. When the pH is over 2.5 and below 3.2, the lowest resolution of Cu–TETA and Cu–CB–DO2A is observed. At a mobile phase pH over 3.2, the resolution of Cu–TETA and Cu–CB–DO2A becomes better but the co-elution of an impurity and the Cu–TETA occurs. Taking these results into consideration, the separation of these Cu–azamacrocyclic complexes is better carried out at pH below 2.5.

Effect of the concentration of the methanol in the mobile phase on the capacity factors

It has been reported that a mobile phase containing less than 10% methanol may not sufficiently wet an octadecylsilated silica-based stationary phase (Li *et al.*, 1996). For this reason, the effect of adding varying amounts of methanol in the citric acid buffered mobile

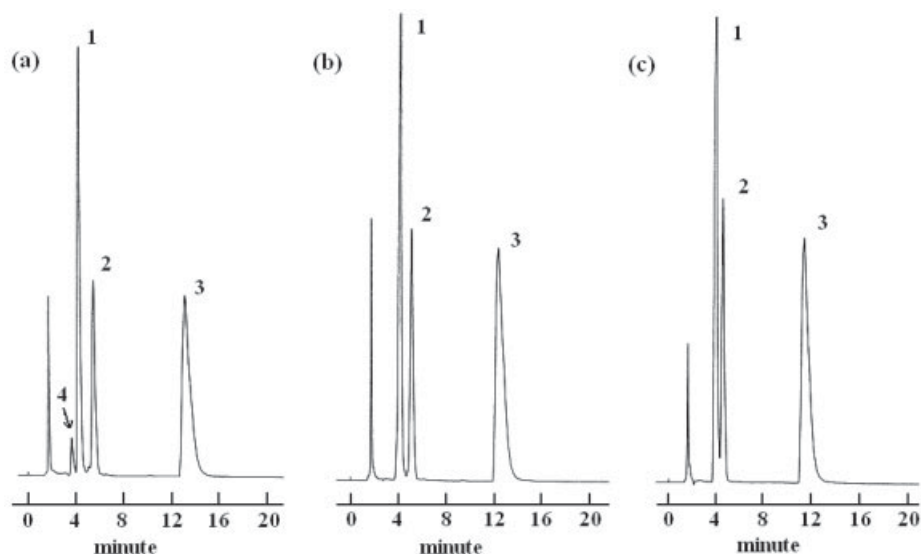


Figure 2. Separations of Cu-CB-TE2A, Cu-TETA, and Cu-CB-DO2A with the use of citric acid buffer at (a) 30, (b) 60 and (c) 150 mM. Mobile phase, citric acid at pH 2.5; stationary phase, Betabasic C_{18} . Peaks: **1**, Cu-CB-DO2A (0.10 mg/mL); **2**, Cu-TETA (0.13 mg/mL); **3**, Cu-CB-TE2A (0.10 mg/mL); **4** = impurity.

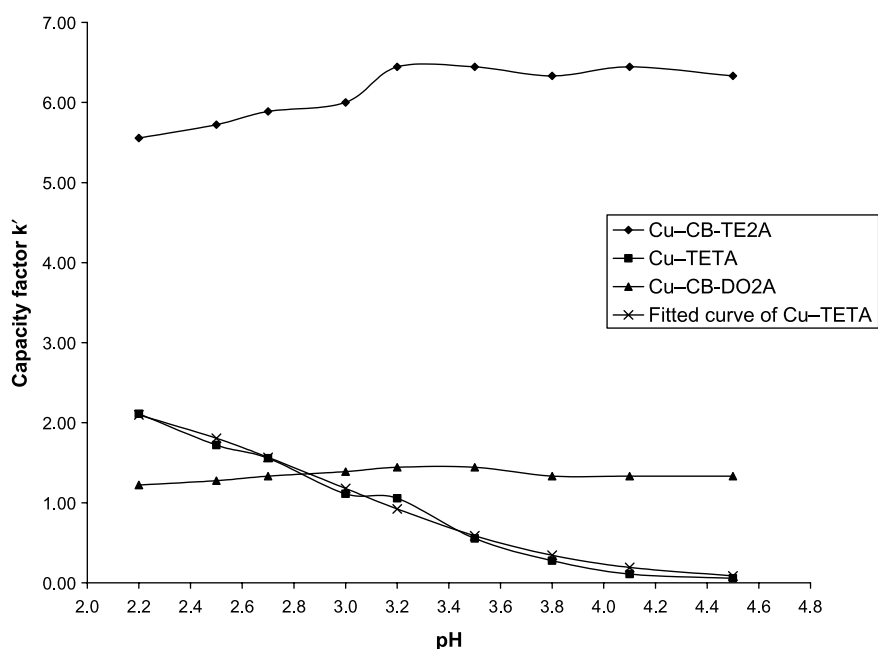


Figure 3. Effect of the pH of the mobile phase on the capacity factor of Cu-CB-TE2A, Cu-TETA and Cu-CB-DO2A. Mobile phase: 30 mM citric acid. Stationary phase, Betabasic C_{18} . The fitted curve of Cu-TETA is plotted according to eq. (1), see the text for details.

phase was evaluated. The effect of the concentration of the methanol in the mobile phase on the capacity factors of the three Cu-azamacrocyclic complexes with the use of a C_{18} column is presented in Fig. 4. It is apparent that the concentration of methanol in the mobile phase greatly affects the capacity factor of these copper complexes. Adding only 2% methanol to the mobile phase results in a reduction in the capacity factor by

half. When a mobile phase containing 10% methanol is used, these copper complexes are hardly retained on the C_{18} column. When the logarithm of the capacity factor is plotted vs the fraction of the methanol in the mobile phase (Fig. 5), the slightly nonlinear trend was similar to that was observed by Hsieh and Dorsey (1993) and attributed to a structural change of the stationary phase within highly aqueous mobile phases.

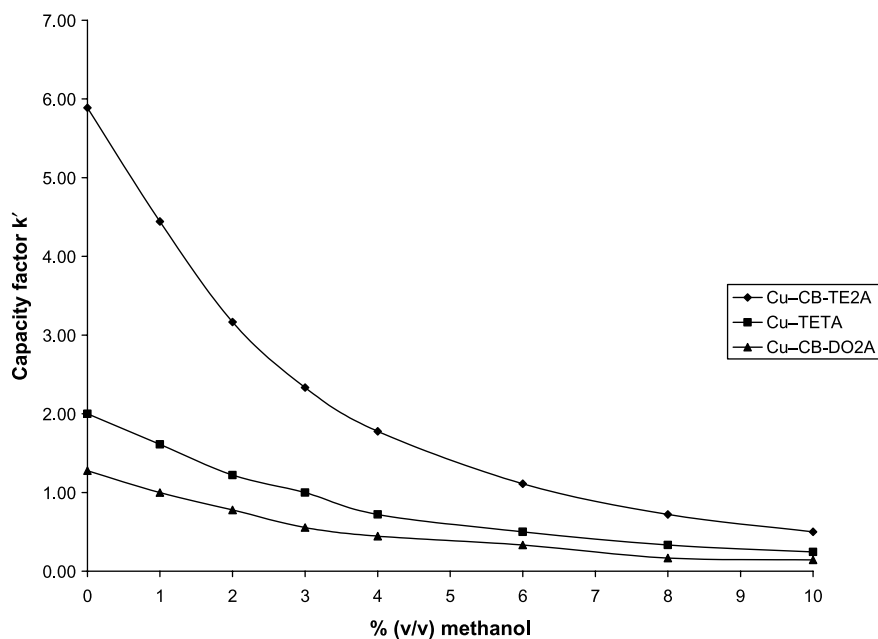


Figure 4. Effect of the concentration of methanol in the mobile phase on the capacity factor of Cu-CB-TE2A, Cu-TETA and Cu-CB-DO2A. Mobile phase, 30 mM citric acid at pH 2.5; stationary phase, Betabasic C_{18} .

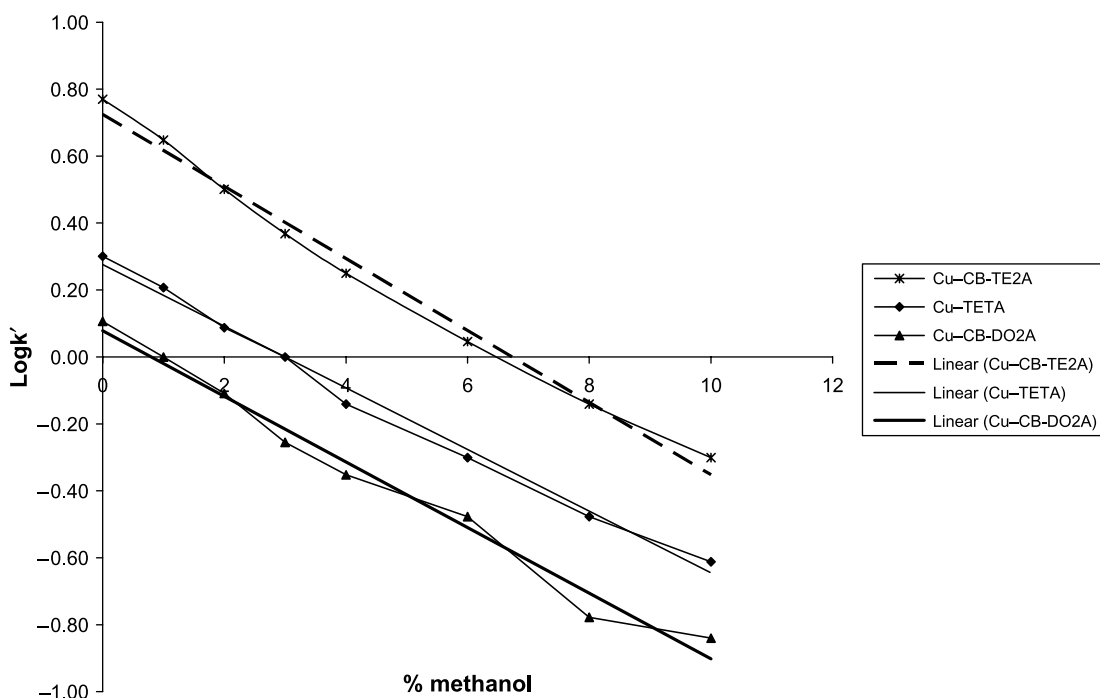


Figure 5. Plots of the $\log(k')$ values of Cu-CB-TE2A, Cu-TETA and Cu-CB-DO2A vs the concentration of methanol and their trends. Mobile phase, 30 mM citric acid at pH 2.5; stationary phase: Betabasic C_{18} .

Comparison of the separations with the use of two different mobile phases on C_8 and C_{18} columns

The separation of the three complexes with the use of 30 mM citric buffer at pH 2.5 on a C_{18} column was

achieved with a better symmetry of the Cu-CB-TE2A peak, as shown in Fig. 2, compared with the severe tailing of the corresponding peak eluted from the C_8 column using 22 mM formic acid as the mobile phase, possibly due to the residual silanols of the C_8 stationary phase (Boswell *et al.*, 2005). To investigate the effects

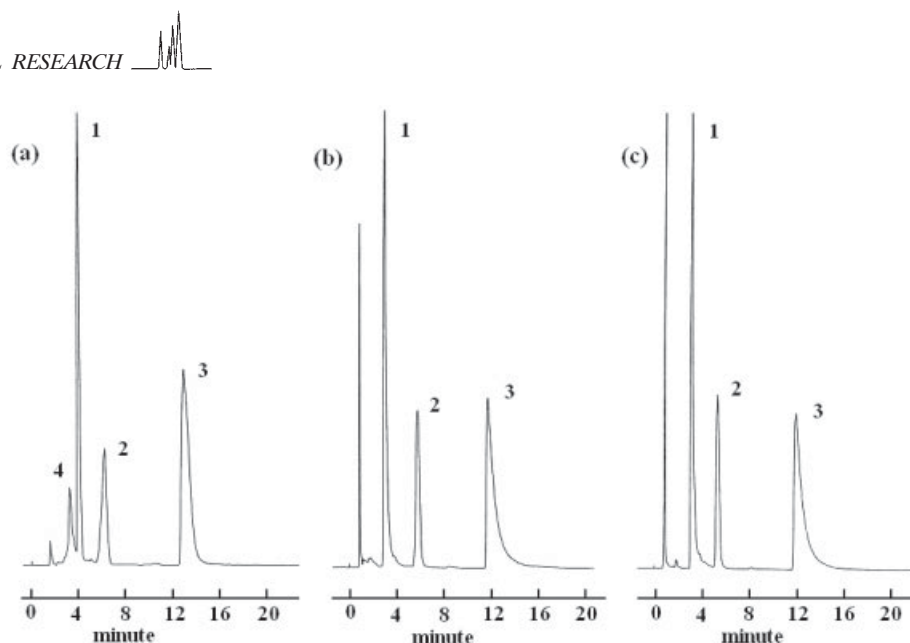


Figure 6. Separations of Cu–CB–TE2A, Cu–TETA and Cu–CB–DO2A with the use of different mobile phases and different columns. (a) Mobile phase, 22 mM formic acid (pH 2.5); column, Betabasic C₁₈; (b) mobile phase, 22 mM formic acid (pH 2.5); column, Zorbax SB–C8; (c) mobile phase, 30 mM citric acid buffer at pH 2.5; column, Zorbax SB–C8. Peaks: **1**, Cu–CB–DO2A (0.10 mg/mL); **2**, Cu–TETA (0.13 mg/mL); **3**, Cu–CB–TE2A (0.10 mg/mL); **4**, impurity.

of different mobile phases and different stationary phases on peak tailing for Cu–CB–TE2A, C₈ and C₁₈ columns were used to compare the profiles of the peak tailing obtained with the use of two different mobile phases. The mobile phases used were 22 mM formic acid (pH 2.5) and 30 mM citric acid buffer at pH 2.5. In addition to the mixture of the three Cu–azamacrocyclic complex standards, an impure Cu–CB–TE2A sample called the ‘crude Cu–CB–TE2A’, recovered from acid decomplexation studies, was also used to evaluate the effects of mobile phase and column on the separations obtained.

The chromatograms showing the separation of the three complexes on the C₁₈ column with the use of 22 mM formic acid (pH 2.5) and on the C₈ column with the use of 22 mM formic acid (pH 2.5) or 30 mM citric acid buffer are presented in Fig. 6. Compared with the separation on a C₁₈ column with the use of 30 mM citric acid buffer at pH 2.5 [Fig. 2(a)], the separation on a C₁₈ column using 22 mM formic acid (pH 2.5) resulted in a longer retention time and slight band-broadening for the Cu–TETA peak [Fig. 6(a)], while the band profiles for Cu–CB–TE2A did not show a significant difference between these mobile phases. When the C₈ column was used, separation of the three complexes with the two different mobile phases, 22 mM formic acid (pH 2.5) and 30 mM citric buffer at pH 2.5, resulted in severe tailing of Cu–CB–TE2A peak, as well as the co-elution of Cu–CB–DO2A with the impurity peak from the Cu–TETA sample [Fig. 6(b,c)]. Thus, it is possible that the severe tailing of the Cu–CB–TE2A peak was not due to

the use of 22 mM formic acid (pH 2.5) but the column employed. The C₁₈ column used here provided better peak symmetry for the Cu–CB–TE2A peak observed than when using the C₈ column.

While the citric acid buffer used at pH 2.5 did not improve the peak symmetry, there are advantages to using citric acid buffer in the mobile phase, such as the capability of maintaining pH over a wider range than for formic acid, potentially resulting in improved reproducibility, and the ability to optimize the separation by adjusting the pH over a wide range. The latter advantage can be observed in Fig. 7 by comparing the separation obtained for the crude Cu–CB–TE2A sample on the C₁₈ column with the use of a mobile phase containing 6 mM citric acid at pH 2.5 and 6.2 as well as 22 mM formic acid (pH 2.5). Impurities were better separated with the use of 6 mM citric acid buffer at pH 6.2 [Fig. 7(a)] than 6 mM citric acid buffer at pH 2.5 [Fig. 7(b)] or 22 mM formic acid [pH 2.5; Fig. 7(c)].

Separation of Cu–azamacrocyclic complexes by ion-pair HPLC using a C₈ column

Although citric acid buffer had some advantages over formic acid as the mobile phase in the separation of Cu–azamacrocyclic complexes by reversed-phase liquid chromatography, two obvious challenges remained:

- (1) The pH of the mobile phase must be lower than the pK_a of acidic solutes to force the association of acid, thereby increasing the retention of the solutes

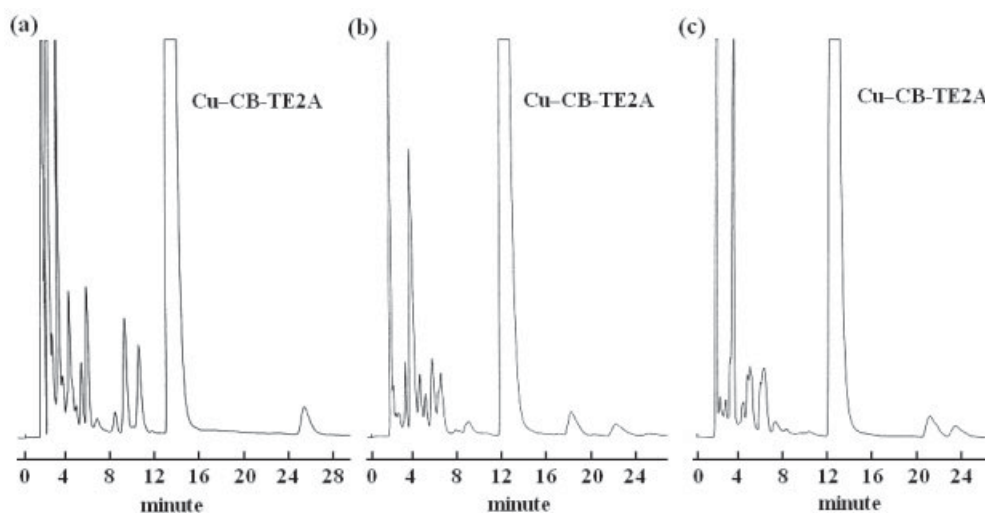


Figure 7. Separations of the crude Cu–CB–TE2A (1.0 mg/mL) with the use of different mobile phases. (a) Mobile phase, 6 mM citric acid buffer at pH 6.2; (b) mobile phase, 6 mM citric acid buffer at pH 2.5; (c) 22 mM formic acid (pH 2.5). Stationary phase, Betabasic C₁₈.

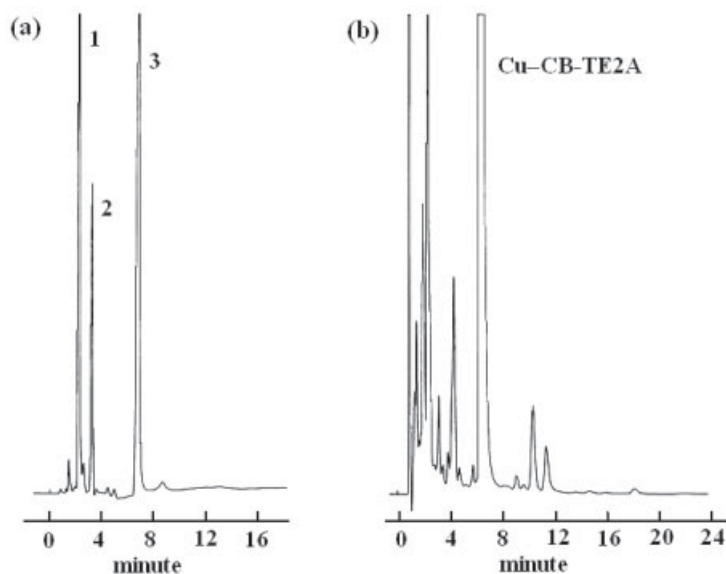


Figure 8. Ion-pair HPLC separations of (a) Cu–CB–TE2A, Cu–TETA and Cu–CB–DO2A; (b) the crude Cu–CB–TE2A (1.0 mg/mL). Mobile phase, 40 mM TEAA at pH 6.3. Stationary phase, Zorbax SB–C₈. Peaks: **1**, Cu–CB–DO2A (0.10 mg/mL); **2**, Cu–TETA (0.13 mg/mL); **3**, Cu–CB–TE2A (0.10 mg/mL).

on the reversed-phase column. If the mobile phase pH required is too low, damage to silica-based alkyl-bonded phases may occur due to the hydrolysis of the siloxane bond.

- (2) An additional requirement may be that the mobile phase buffer be volatile so that it will be compatible with LC–MS, a technique which has been widely used in drug discovery and development.

Ion-pair reversed-phase liquid chromatography (Hearn, 1985) has the advantage of simultaneously separating

ionic and neutral solutes with the use of a reversed-phase column and a volatile ion-pair reagent in the mobile phase at a moderate pH. Therefore this method was employed as an alternative for separating the Cu–azamacrocyclic complexes. TEAA has been used as a volatile ion-pair reagent for the analysis of nucleic acids by LC–MS (Huber and Krajete, 1999; Premstaller *et al.*, 2000). The separation of the Cu–azamacrocyclic complexes by ion-pair HPLC on the C₈ column with the use of 40 mM TEAA in the mobile phase at pH 6.3 is presented in Fig. 8(a). The complexes are well

separated from several small peaks which were not previously observed under reversed-phase conditions (Fig. 6). Compared with the separation obtained using reversed-phase conditions on a C₈ column, shorter retention times were observed for the three Cu complexes when using ion-pair conditions and the same C₈ column. This is attributed to the competition between the solutes and the hydrophobic triethylammonium for the stationary phase, hence the reduced hydrophobic interaction between the reversed-phase and the solutes. The crude Cu-CB-TE2A sample was also used to evaluate the separations produced by the ion-pair reversed-phase HPLC method. The separation of the crude Cu-CB-TE2A with the use of 40 mM TEAA in the mobile phase at pH 6.3, presented in Fig. 8(b), shows a similar resolution of the impurity peaks as was obtained using 6 mM citric acid buffer in the mobile phase at pH 6.2 [Fig. 7(a)].

Estimation of the pK_as of Cu-TETA using reversed-phase chromatographic data

Since two of four carboxylic acid arms of TETA complex with the Cu²⁺ ion, Cu-TETA is considered to be a diprotic acid (Fig. 1). As shown in Fig. 3, the capacity factor of Cu-TETA is dependent on pH. This relationship can be described using an equation for the capacity factor of diprotic acids as a function of the concentration of protons (H⁺) in the mobile phase (Horváth *et al.*, 1977):

$$k' = \frac{k'_0 + k'_{-1} \frac{K_{a1}}{[H^+]} + k'_{-2} \frac{K_{a1}K_{a2}}{[H^+]^2}}{1 + \frac{K_{a1}}{[H^+]} + \frac{K_{a1}K_{a2}}{[H^+]^2}} \quad (1)$$

where k'_0 , k'_{-1} and k'_{-2} are the capacity factors of the undissociated, half-dissociated, and fully dissociated diprotic acid and K_{a1} and K_{a2} are the corresponding acid dissociation constants in the mobile phase, respectively. These five parameters k'_0 , k'_{-1} , k'_{-2} , K_{a1} and K_{a2} , can be determined by fitting the appropriate model, i.e. eq. (1), by nonlinear regression (Rusling and Kumosinski, 1996), to a series of capacity factor values measured at different mobile phase pH for the solute. The statistical software JMP was used to perform the nonlinear fitting of the data. In order to reduce the uncertainties in the estimation of five parameters by nine data points when JMP performs nonlinear fitting, some parameters were locked. The parameters being locked were k'_0 , k'_{-1} and k'_{-2} , because k'_0 and k'_{-2} can be easily estimated as $k'_0 \approx 2.4$ by extrapolating the curve to pH = 0 where Cu-TETA is undissociated, and $k'_{-2} \approx 0$ by extrapolating the curve at pH above 4.5 where Cu-TETA is almost fully dissociated, while k'_{-1} is estimated as having a value between k_0 and k_{-2} . Thus, fitting of

the data was performed by varying the values of k_0 , k_{-1} and k_{-2} to calculate different values of the sum of square error (SSE), which is an indicator of how well the line fits. When k'_0 , k'_{-1} and k'_{-2} are locked at 2.6, 1.1 and 0.01, respectively, a minimum value of SSE is achieved and the estimated values of K_{a1} and K_{a2} are obtained: $pK_{a1} = 2.32 \pm 0.09$ and $pK_{a2} = 3.19 \pm 0.05$. These values of the five parameters are employed to plot the corresponding fitted curve shown in Fig. 3 with the use of eq. (1). Clarke and Martell (1991) reported a pK_{a1} of 2.50 ± 0.01 and a pK_{a2} of 3.91 ± 0.01 for Cu-TETA. These pK_a values were determined by potentiometric titration in aqueous KCl solution (0.1 M, 25.0°C). They also reported the values for $pK_{a1} = 2.90 \pm 0.03$ and $pK_{a2} = 3.68 \pm 0.03$ for Cu-TETA based on data from Delgado and Frausto Da Silva (1982), which were determined by potentiometric titration in aqueous KNO₃ solution (0.1 M, 25.0°C; Delgado and Frausto Da Silva did not report directly the pK_{a1} and pK_{a2} of Cu-TETA, but these two values can be derived mathematically from their data). Our pK_{a1} value is close to the result reported by Clarke and Martell (0.2 logarithm unit difference), while pK_{a2} is close to Delgado and Frausto Da Silva's result (0.5 logarithm unit difference). The ionic strength of the 30 mM citric acid-buffered mobile phase we used is different from the ionic strength of the solutions for the potentiometric titration reported, which may result in the pK_a values of the Cu-TETA-based on HPLC data differing from the values obtained by potentiometric titration. It is noted that the other parameters such as k'_0 , k'_{-1} and k'_{-2} in eq. (1) are constants which will be determined by performing nonlinear fitting. However, previous discussion has shown that the capacity factors of the two neutral complexes, Cu-CB-TE2A and Cu-CB-DO2A vary slightly as the pH increases (Fig. 3). The same effect may also be applied to the undissociated, half-dissociated, and fully dissociated Cu-TETA, which means k'_0 , k'_{-1} and k'_{-2} in eq. (1) may not be constant when performing nonlinear fitting to obtain the values of pK_{a1} and pK_{a2} , and may introduce some uncertainty into the results of pK_{a1} and pK_{a2} . There are also outliers in our experimental data that may reduce the accuracy of the nonlinear fitting.

Estimation of the lipophilicity of the complexes by reversed-phase HPLC

Although the terms 'lipophilicity' and 'hydrophobicity' are frequently considered to be synonymous and both are used in the literature, their scientific meanings are quite different. The following definitions have been recommended by IUPAC (1998).

- 'Lipophilicity represents the affinity of a molecule or a moiety for a lipophilic environment. It is

commonly measured by its distribution behaviour in a biphasic system, either liquid–liquid (e.g. partition coefficient in octan-1-ol/water) or solid–liquid (retention on reversed-phase high performance liquid chromatography (RP-HPLC) or thin-layer chromatography (TLC) system).’

- ‘Hydrophobicity is the association of non-polar groups or molecules in an aqueous environment which arises from the tendency of water to exclude non-polar molecules.’

Discussions of the difference between these two terms can be found in the literature (Pliska *et al.*, 1996; Nasal *et al.*, 2003). It is well known that lipophilicity plays an important role in the physico-chemical properties and pharmacokinetic behavior of pharmaceuticals, and it is usually expressed as the octanol–water partition coefficient ($\log P$; Pliska *et al.*, 1996), which has traditionally been measured by the so-called ‘shake-flask’ method. Estimating the lipophilicity of a solute by reversed-phase liquid chromatography is based on developing a relationship between the logarithm of the partition coefficient and the logarithm of the capacity factor of the solute described by the following equation (Braumann, 1986):

$$\log P = a \log(k') + b \quad (2)$$

The values of a , $\log(k')$ and b are highly dependent on the concentration of the organic modifier in the mobile phase. For this reason, the capacity factor obtained using a 100% aqueous mobile phase, $\log(k'_w)$, is often used to replace $\log(k')$ in eq. (2). In most cases, the use of a 100% aqueous mobile phase is experimentally unrealistic since it is too weak to elute the solutes in a reasonable period of time. For this reason, $\log(k'_w)$ values are often obtained by extrapolating from the plot of $\log(k')$ vs the fraction of organic modifier to 0% organic modifier by fitting the experimental data to a suitable model. Octadecyl stationary phases are most often used to measure the $\log(k'_w)$ values. However, other stationary phases have also been used for this purpose. Several reviews (Lambert, 1993; Dorsey and Khaledi, 1993; Nasal *et al.*, 2003) have discussed the effect of the stationary phase on the determination of $\log(k'_w)$. It is generally agreed that, with the use of methanol–water mobile phases, the capacity factors obtained using a C_{18} stationary phase correlate well with literature values of octanol–water partition coefficients. Although the regression coefficients for the correlation between $\log P$ and $\log(k'_w)$ vary for different solutes and under different chromatographic conditions, Braumann (1986) concluded that the regression coefficients approach 1.0 and 0.0 for the slope and intercept, respectively, resulting in $\log P = \log(k'_w)$. Although views differ over whether $\log(k'_w)$ and $\log P$ are identical, $\log(k'_w)$ has become the most widely used

chromatographic parameter of lipophilicity (Kaliszan, 1998; Berthod and Carda-Broch, 2004; Tate and Dorsey, 2004).

The $\log(k'_w)$ values of the neutral complexes, Cu–CB-DO2A and Cu–CB-TE2A, were obtained directly with the use of a totally aqueous mobile phase using an octadecyl stationary phase, as shown in Fig. 5. The $\log(k'_w)$ values for Cu–CB-DO2A and Cu–CB-TE2A were found to be 0.11 and 0.77, respectively. For an ionic compound, the $\log(k'_w)$ value of the undissociated form is generally considered (Dorsey and Khaledi, 1993). Therefore, the $\log(k'_w)$ value of the ionic complex, Cu–TETA, is given by the logarithm of the capacity factor of undissociated Cu–TETA, $\log k'_0 \approx \log 2.6 = 0.41$, where k_0 is estimated by nonlinear regression as previously discussed. This study provides a simple and reasonable method for estimating the relative lipophilicity of the three complexes. Additional studies would be required to explore the relationship between $\log(k'_w)$ and the structural properties of the Cu–azamacrocyclic complexes in terms of quantitative structure–retention relationships or to correlate $\log(k'_w)$ with their relevant bio-partitioning processes.

CONCLUSION

The separation of three Cu–azamacrocyclic complexes, Cu–CB-TE2A, Cu–TETA and Cu–CB-DO2A, was achieved by reversed-phase HPLC with the use of 30 mM citric acid buffer in the mobile phase at a pH below 2.5. Addition of methanol in the mobile phase greatly affected the retention of these polar copper complexes. A better separation of the crude Cu–CB-TE2A sample was achieved with the use of 6 mM citric acid buffer, pH 6.2, than when using 22 mM formic acid (pH 2.5). Differences in tailing profiles observed for Cu–CB-TE2A appeared to be the result of differences in the interactions of Cu–CB-TE2A with the C_8 and C_{18} stationary phases. Ion-pair HPLC was shown to be an effective alternative for the separation of these complexes with the use of 40 mM TEAA as the ion-pair reagent in the mobile phase at pH 6.3. Finally, the pK_a s of the acidic solute, Cu–TETA, and the lipophilicity parameter, $\log(k'_w)$, of three Cu–azamacrocyclic complexes were estimated with the use of reversed-phase HPLC.

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