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Chromatographic studies and analytical methods development for selected tetraazamacrocycles and their copper(II) complexes

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CHROMATOGRAPHIC STUDIES AND ANALYTICAL METHODS DEVELOPMENT FOR SELECTED TETRAAZAMACROCYCLES AND THEIR Cu(II) COMPLEXES

BY

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DISSERTATION

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DEDICATION

I dedicate this dissertation to my wife Orjana for her unconditional love and help through the hard times in my life. In addition I dedicate this work to my grandaunt Meropi Face who instilled in me the passion for learning and the desire to succeed.

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ABSTRACT

CHROMATOGRAPHIC STUDIES AND ANALYTICAL METHODS DEVELOPMENT FOR SELECTED TETRAAZAMACROCYCLES AND THEIR Cu(II) COMPLEXES

BY

Ilia Terova

University of New Hampshire, December, 2010

Cu(II)-azamacrocyclic complexes are of current interest due to their potential application as copper-64 radioisotope-based imaging and therapeutic agents. The development of separation conditions is important in evaluating their purity as well as assessment of their physical properties including conditional formation constants, acid dissociation constants and lipophilicity, which can have a major bearing on the *in vivo* behavior of these complexes. This study evaluates the chromatographic properties of a series of copper (II) complexes of chelating cross-bridged tetraamine ligands with methanephosphonate arms having different ring sizes (12- and 14-membered) and different pendant arm moeties. Cu-CB-DO2P^{OEt} and Cu-CB-TE2P^{OEt} are two complexes with different ring size but the same pendant arm. Cu-CB-TE2P^{OEt} and Cu-CB-TE2P are two complexes with different pendant arms but the same ring size. A model study proved the viability of chromatographic methods as a means of measuring the conditional formation constant of copper(II) complexes. The conditional formation constant for Cu-cyclen was evaluated through a competition reaction with cyclam. The estimation of the pK_as for a copper (II) complex with methanephosphonate pendant arms is reported. The role of the stationary phase in the determination of the pK_a values was investigated using

different reversed phase stationary phases. In addition the lipophilicity parameter for a series of methanephosphonate copper (II) complexes (Cu-CB-TE2P, Cu-CB-TE1A1P, Cu-CB-DO2P^{OEt} , and Cu-CB-TE2P^{OEt}) was estimated. The chiral separation for polycyclic tetramines including dibenzocyclam bisaminal, CB-cyclam, and dimethyl dibenzo CB-cyclam was achieved on chiral polysaccharide stationary phases. Successful enantiomeric separation methodology allows for evaluation of the enantiopurity of the resolved ligands. In addition the evaluation of thermodynamic data such as the barrier of racemization for CB-cyclam was investigated.

CHAPTER I

INTRODUCTION

1.1 Background

Chromatography has become one of the most widely used analytical techniques over the past several decades. It has a broad range of applications from analyzing the purity of active ingredients in pharmaceutical compounds, to detecting the presence of illegal drugs in urine or blood, and detecting levels of contaminants in water.[1-4] Gas chromatography and liquid chromatography are the two techniques that are often employed to produce such separations. The physical properties of the analytes such as boiling point, ease of derivatization, and interactions of the analytes with the stationary phases are some of the properties that determine which type of chromatography is ideal.[4-6] One of the most commonly used types of chromatography is high performance liquid chromatography (HPLC).[1]

HPLC allows for the analysis of samples that have high boiling points or are thermally labile offering advantages over gas chromatography. Although it is often possible to derivatize non-volatile samples in order to analyze them by GC; over eighty percent of separations are carried out using HPLC.[4] HPLC can be coupled with a multitude of detectors including mass spectrometer, evaporative light scattering detector, fluorescence, and Ultraviolet-Visible (UV-Vis) absorbance detectors. The HPLC studies

performed during this research utilized both UV-Vis and Circular Dichroism (CD) detection. The CD detector is used for chiral separations as the two enantiomers interact with circularly polarized light differently. CD detectors can help in the evaluation of enantiomeric purity once a separation has been obtained.

The analytes of interest are tetraamine ligands and their copper(II) metal complexes. Depending on the pH of the solution they may exist as anions, cations or be neutral species.[7-13] These analytes have different hydrophobicities and charges. The structures of two ligands, a synthetic precursor and two copper(II) cross-bridged complexes are presented in Figure 1.1. All the analytes in Figure 1.1 are neutral with the exception of Cu-CB-TE1A which is a cation.

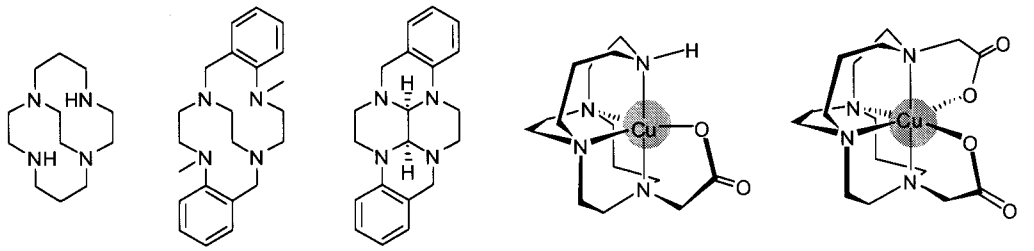


Figure 1.1. Structures of (a), CB-cyclam; (b), dibenzo dimethyl CB-cyclam; (c), dibenzo cyclam bisaminal; (d), Cu-CB-TE1A and (e), Cu-CB-TE2A.

Different modes of separations were utilized including reversed phase HPLC (RP-HPLC), normal phase HPLC, ion-pair, ion-exchange, and chiral HPLC. In recent years RP-HPLC has become the most popular technique for analytical separations for a wide range of compounds.[4-6] One of the reasons for its extensive use is due to the wide

variety and availability of stationary phases. Some of the most commonly used stationary phases in RP-HPLC are octyl (C_8), octadecyl (C_{18}), phenyl and cyano moieties bonded typically on silica particles. In RP-HPLC, differences in the retention times of the analytes are due mainly to differences in hydrophobicity of the analyte making it ideal for the analysis of both non-polar and polar compounds.[14-17] Among other factors that influence retention in RP-HPLC are temperature, mobile phase additives, pH, and the charge of the analyte.

Highly efficient separations in HPLC can be obtained using columns packed with small diameter particles operated at high pressures, employing mobile phases of low viscosity. In chromatography, column efficiency can be evaluated by measuring the theoretical plate height (H) and the number of theoretical plates (N). These parameters, N and H, are based on the plate model of chromatography.[2] The plate model assumes that the column can be visualized as being divided into a number of discrete volume elements or imaginary sections called "plates". At each plate the partioning of the solute between the mobile and stationary phase is assumed to be rapid with equilibrium being reached before the solute moves on to the next plate. The plate model is useful for characterizing the efficiency of distillation columns and liquid extractors but has its limitations when applied to chromatographic processes.[2,4-6] However, the measured quantities H and N are useful parameters for characterizing chromatographic efficiency.

RP- stationary phases tend to have reasonably high plate numbers.[4-6] The most commonly used RP-HPLC stationary phase supports have a diameter of 5μm at this time. Recently there has been an extensive push toward producing stationary phases that have small particle diameters. The 2μm and sub-2μm particle are available for different

stationary phases. There are C_{18}, C_8, cyano and phenyl based stationary phases on these smaller particles, which make it possible to utilize different types of solute-stationary phase interactions to achieve the separation. These stationary phases produce even higher plate numbers. Due to the higher pressures generated, separations using these 2μm and sub-2μm stationary phases are known as Ultra High Performance Liquid Chromatography (UPLC).[17-20] In the future this type of smaller particle stationary phase can be used to achieve separations in a shorter amount of time and with lower cost since the smaller columns would also require less solvent and generate less waste.

1.2 Research goals

In this research, separation conditions were developed for a variety of copper cross-bridged complexes and their tetraazamacrocyclic ligands. One of the goals is to understand the correlation between the structural properties of carboxymethyl pendant arm cross bridged complexes and their observed chromatographic behavior (Figure 1.2).

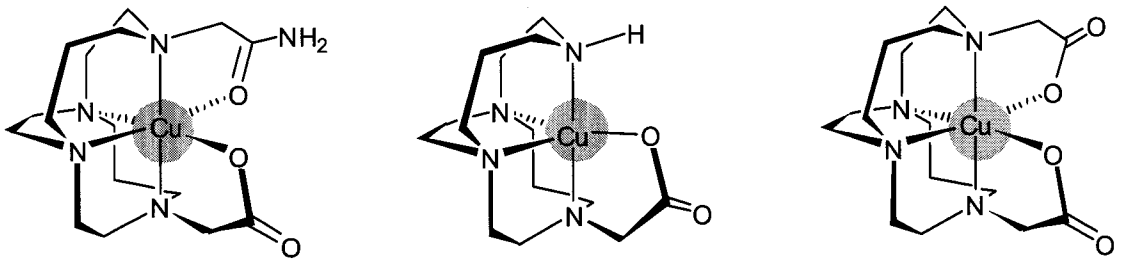


Figure 1.2. Structures of (a), Cu-CB- TEAMA; (b), Cu-CB-TE1A; and (c), Cu-CB-TE2A

Three different types of stationary phases were used to elucidate this correlation: cation exchange, porous graphitic carbon and C_{18}. The use of cation exchange stationary

phases allowed the separation of Cu-cyclen, Cu-cyclam, Cu-CB-cyclen and Cu-CB-cyclam shown in Figure 1.3.

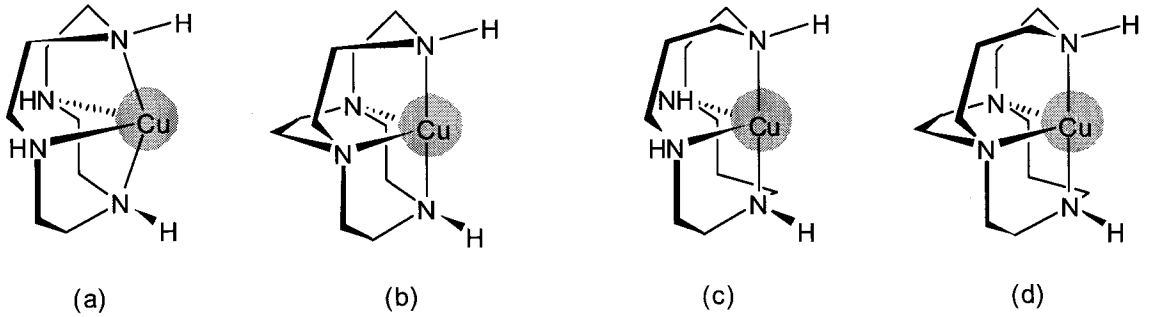


Figure 1.3. Structures of (a), Cu-cyclen; (b), Cu-CB-cyclen; (c), Cu-cyclam; and (d), Cu-CB-cyclam

The behavior of the charged complexes, Cu-CB-TE1A and Cu-CB-TEAMA, on a porous graphitic carbon stationary phase provided a better understanding of the retention mechanisms. The effect of different mobile phases and the use of the best stationary phase to achieve a separation will be discussed in greater detail. The chromatographic methods developed and conditions investigated as part of this research can be used to monitor reactions, to purify analytes and to assess physico-chemical properties that are not easily measured through other methods.

The evaluation of conditional formation constants is also important since it provides insights into the *in-vivo* stability of the copper(II) complexes. Another goal of this research is to prove the viability of HPLC as a method for the measurement of conditional formation constants for copper(II) complexes with tetraazamacrocyclic ligands. Competition reactions were used to gain insights into the formation constants of Cu-CB-cyclam and Cu-CB-TE2A.

Acid dissociation constants for Cu-CB-TE2P, a newly synthesized copper(II) complex, were measured by use of HPLC. The structures of Cu-CB-TE2P and a related copper(II) complex are shown in Figure 1.4. The pH dependence of the Cu-CB-TE1A1P complex (Figure 1.4) on a C_{18} stationary was also investigated.

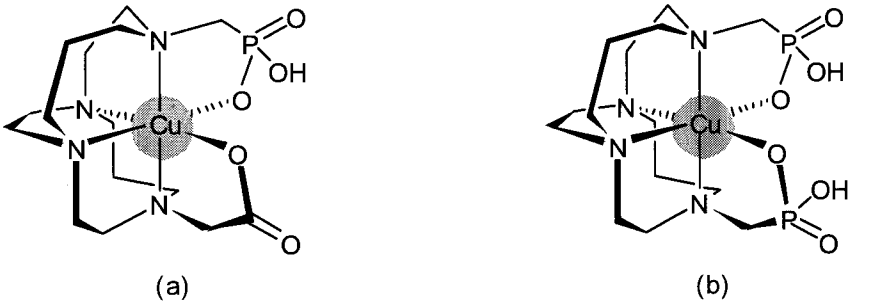


Figure 1.4. Structures of (a), Cu-CB-TE1A1P and (b), Cu-CB-TE2P

The importance of stationary phase selection as it affects the measurement of acid dissociation constants was evaluated. The lipophilicity parameter for selected copper complexes was measured using aqueous mobile phases and a C_{18} stationary phase. A comparison of the chromatographic behavior of copper complexes on several stationary phases allowed for an elucidation of the retention mechanism for these complexes.

An additional goal of this research was to develop conditions which provide chiral separations for CB-cyclam, dimethyl dibenzo CB-cyclam and dibenzobisaminal (Figure 1.1). Three different chiral stationary phases were evaluated in order to gain a better understanding of chiral discrimination for each cross-bridged tetraazamacrocycle. Several additives and mobile phase compositions were investigated in order to optimize the resolution of the enantiomers. The chiral HPLC separation conditions allow for easy evaluation of the racemization barrier as well as the enantiomeric purity of the material

prior to use in a reaction. The enantiopure ligand will yield an enantiopure metal complex whose behavior can be studied and compared to that of the racemate.

1.3 Developing HPLC methods for measuring conditional formation constants of selected copper(II) tetraazamacrocyclic complexes

Polyamine macrocycles are able to bind tightly to a variety of transition metal cations. The parent macrocycles, cyclam and cyclen (Figure 1.5), can be modified by the addition of carboxylate pendant arms to the secondary nitrogens to obtain new ligands with different chelating properties such as H_4DOTA and H_4TETA, which are shown in Figure 1.5.[21-23] The functional group and length of these pendant arms can be selectively adjusted to fine tune the properties of the metal complex.

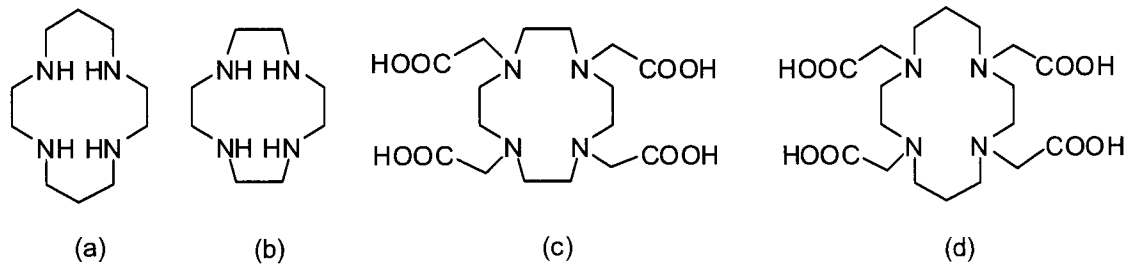


Figure 1.5. Structures of (a), cyclam; (b), cyclen; (c), H_4DOTA; and (d), H_4TETA

Metal complexes of these ligands exhibit enhanced thermodynamic stability which is attributed to the macrocyclic effect.[23-25] This effect can be explained both in terms of a larger enthalpy and a smaller entropy of complexation compared to similar acyclic analogues. Due to their thermodynamic stability and possible *in vivo* applications there has been an increased interest in macrocyclic tetraamines as chelating agents in recent decades. For instance tetraazamacrocyclic DOTA has been used in

radiopharmaceutical applications including labeling monoclonal antibodies with radioactive metals for cancer diagnosis and therapy. [26,27] In addition DOTA has also been used with paramagnetic cations for magnetic resonance imaging .[26,27]

Weisman, Wong and co-workers were the first to report a new class of novel cross-bridged tetraamine ligands featuring an ethylene bridge linking two nonadjacent nitrogens. [7-9] These cross-bridged ligands can be further functionalized with various pendant arms (Figure 1.6) to allow for the fine tuning of metal coordination. For example CB-TE2A, and CB-TE2P feature an ethylene bridge with short and long arm carboxylate and methanephosphonate arms respectively (Figure 1.6).

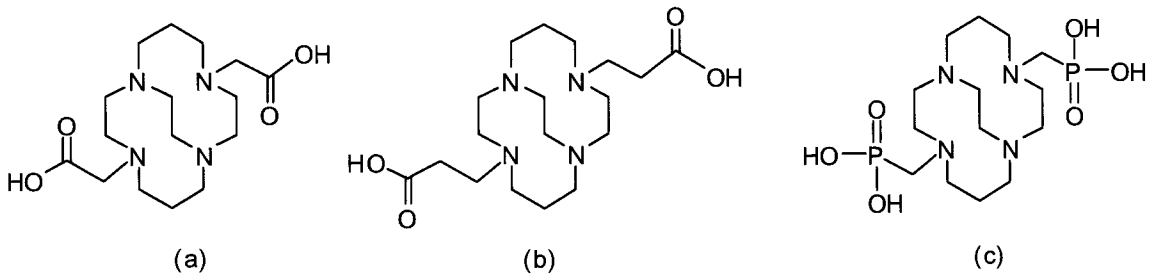


Figure 1.6. Structures of modified tetraazamacrocycles (a), H_2-CB-TE2A; (b), H_2-CB-TE2LA; and (c), H_4-CB-TE2P

Several copper (II) complexes of the cross-bridged tetramines have been synthesized. Development of chromatographic separation conditions of these copper complexes allows for a fast and efficient way to assess reaction completion or to evaluate the purity of the product. A further goal of this research was to use the chromatographic methods developed as part of this work to determine the conditional formation constant (K'_f) for selected copper complexes.

The separation of metal complexes by HPLC has evolved over the past decades.[28] Several chromatographic modes including normal phase, ion exchange, ion pair chromatography, and reverse phase (RP) chromatography have been employed to separate, purify and characterize metal complexes.[28-31] Koo investigated the chromatographic behavior of metal carbonyl complexes using normal phase chromatography. The ligand 1,1' – bis(diphenylphosphino)ferrocene is presented in Figure 1.7. The metal carbonyl complexes, formed from chromium, molybdenum, or tungsten and this ligand were separated using a mobile phase of 98/2 (v/v) hexane/chloroform on a stationary phase consisting of silica particles.[29] The separations developed by Koo allowed for the monitoring of reaction completion.

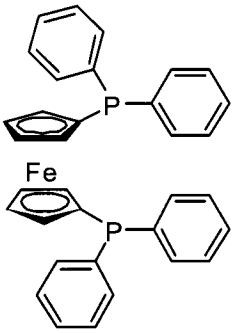


Figure 1.7. The structure of 1,1' – bis(diphenylphosphino)ferrocene

Charged metal complexes have been separated using ion pair and ion exchange chromatography. For example, charged platinum-thiourea complexes were separated by ion pair chromatography using acetonitrile/water as the mobile phase with sodium dodecyl sulfate as the ion pairing reagent.[33] According to literature reports organo-metallic complexes have been studied using RP conditions with C_{18} being the most commonly used stationary phase for the separation of amine-based metal

complexes.[2,32,33] Complexes of 1-(2-pyridilazo)-2-naphthol and 1-(2-thiazolylazo)-2-naphthol, with divalent metals, such as palladium, copper, nickel, cobalt, or trivalent metals, such as iron, rhodium, and cobalt, have been separated by RP chromatography.[32,33] The structures of these ligands are given in Figure 1.8. The separations were carried out using a C_{18} stationary phase with aqueous acetonitrile mobile phases containing acetate to ascertain the effect of pH on retention.[33]

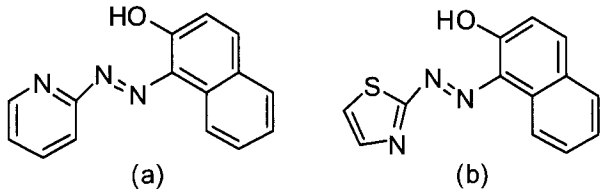


Figure 1.8 The structures of (a), 1-(2-pyridilazo)-2-naphthol and (b), 1-(2-thiazolylazo)-2-naphthol

Complexes of divalent copper or nickel and di-bis(salicylaldehyde)stilbenediimine have been separated by RP HPLC.[34] The synthesis of $[Ru(cyclam)Cl_2]Cl$ was monitored by RP HPLC using a C_{18} stationary phase and isocratic elution with 45/55 (v/v) methanol/water containing 0.1% trifluoroacetic acid.[35]

The most common HPLC conditions for the separation of metal complexes with polyamine ligands also utilize RP stationary phases.[30-35] Polyamine ligands DOTA-BA and DOTA-MBA have been used to form complexes with metals including indium and yttrium. The structures of these ligands are presented in Figure 1.9.[26,27]

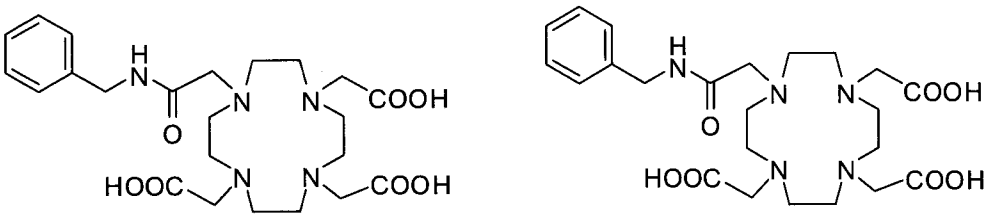


Figure 1.9. Structure of (a), DOTA-BA and (b), DOTA-MBA

Liu and coworkers reported purifying several indium (III) complexes including In-DOTA-BA and In-DOTA-MBA using a reversed phase C_{18} stationary phase by gradient elution. The initial conditions were 30% of A (0.1% TFA in water)/70% of B (0.1% TFA in acetonitrile) increasing linearly to 40% A over 20 minutes, and holding at 40% A for an additional 6 minutes.[26] Gradient elution RP HPLC was also used to analyze other indium (III) complexes with DOTA derivatives, using a mobile phase gradient (8-10 % B over 18 minutes, solvent A = 25 mM acetate buffer, pH 6.8, solvent B = acetonitrile).[27]

Two copper complexes more closely related to this work, Cu-CB-DO2A and Cu-CB-TE2A, have been analyzed by RP-HPLC by Shen.[35-36] The structures of the metal complexes Cu-CB-DO2A and Cu-CB-TE2A are shown in Figure 1.10.

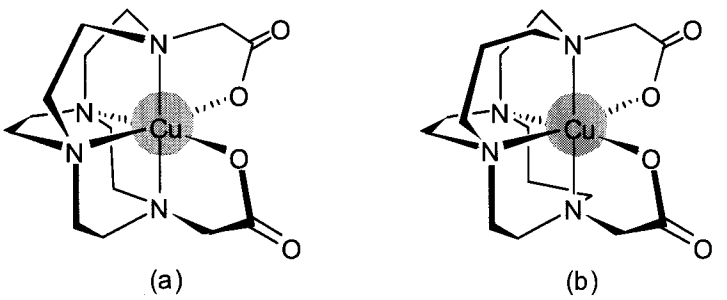


Figure 1.10. Structures of (a), Cu-CB-DO2A and (b), Cu-CB-TE2A complexes

Shen developed separation conditions for several related complexes including Cu-TETA, Cu-CB-TE2LA and Cu-CB-DO2LA utilizing a methanol/water mobile phase on a Betabasic C_{18} stationary phase. The pH of the mobile phase was maintained at approximately 2.3 for these separations in order to ensure that the Cu-TETA complex was neutral. Another type of chromatography used by Shen to achieve the separation of several copper tetraazamacrocyclic complexes (Cu-TETA, Cu-CB-TE2A, and Cu-CB-DO2A) was ion pair chromatography. Triethylammonium acetate was used as the ion pairing reagent (40mM; pH 6.3) with a C_8 stationary phase.[35] The separation of selected complexes (Cu-CB-TE2A, Cu-CB-DO2A, Cu-CB-TE2LA, and Cu-CB-DO2LA) was also evaluated using a porous graphitic carbon (PGC) stationary phase. PGC was found to provide enhanced interactions with these analytes requiring greater amounts of organic modifiers, methanol, to be used in the mobile phase.

In this study, the chromatographic behaviors for several additional copper tetraazamacrocycles including Cu-CB-TE1A, Cu-CB-cyclam, Cu-cyclam, Cu-CB-TEAMA, Cu-CB-cyclen, and Cu-cyclen were evaluated (Figures 1.2 and 1.3). All of these complexes are cations that have either a +1 or +2 charge within the pH range of 2.0 to 8.0. Cu-cyclen, Cu-cyclam, Cu-CB-cyclen, and Cu-CB-cyclam, are +2 charged complexes, therefore their separation was achieved using a strong cation exchange stationary phase instead of a C_{18}. Cu-CB-TE1A and Cu-CB-TEAMA, which are +1 charged complexes, were also separated on the strong cation exchange stationary phase. The chromatographic behavior of these complexes was investigated on the PGC stationary phase as well. It is possible to retain charged analytes on the PGC stationary phase due to polar and electronic interactions between the solutes and the stationary

phase in addition to hydrophobic interactions. Therefore it is reasonable to expect that the PGC stationary phase can be used as an alternative to ion exchange chromatography for the separation of charged copper tetraazamacrocyclic complexes.

In theory, the chromatographic conditions developed for the separation of these copper(II) complexes can be used to determine their K'_fs . The K'_f is dependent on pH, temperature and ionic strength.[37,38] Varying any of these parameters will result in corresponding changes to the value of the formation constant. The K'_f of a complex is an effective measure of the affinity of a ligand (L) for a metal ion (M^{n+}). The K'_f is a quantitative indication of the success or failure in ligand design.[37] For this reason it is important to measure the K'_f for Cu-cyclam, Cu-cyclen, Cu-CB-cyclam, Cu-CB-cyclen and Cu-CB-TE2A. Knowing the K'_f for each of the copper complexes allows the prediction of whether or not these complexes are going to be stable *in vivo*. There are several methods that can provide accurate values for K'_f including potentiometry, spectrophotometry, polarography, and chromatography.[39]

The most common approach for determining the conditional formation constant of a metal complex utilizes potentiometry.[39] There are two approaches to measuring the conditional formation constants when using potentiometry; one is to use a metal specific ion electrode and the other is to use a glass electrode for measuring the hydrogen ion concentration. The metal specific electrode allows for the direct measurement of free metal ion in the solution and then the amount of complex formed can be calculated. The amount of complex formed is inferred by the mass balance for the metal ion. The total concentration of metal in the solution is the sum of the free metal in solution and the metal complex. The ligand is added using a burette so that the concentration of ligand in

solution can be calculated. The amount of any free ligand in solution is inferred from the mass balance for the total ligand concentration. In the case of the glass electrode a standard base is added to a solution of the acidic ligand in the absence and presence of a known total metal ion concentration. One of the limitations for the potentiometric technique is the inability of the base to neutralize one or more of the donor groups on the ligand in the absence of the metal ions. The measurement of K'_fs is thus limited to a pH range of 2-12 where the glass electrode is accurate. For a solution with a pH greater than 12 the glass electrode, which measures the hydronium ion activity suffers from interferences due to the presence of hydroxide ions in concentration 0.01M or greater. Tetraazamacrocyclic ligands including TETA and CB-TE2A are considered "proton sponges". These two ligands will require a pH greater than 12 to achieve neutralization. Therefore, the potentiometric technique has limitations when it comes to measuring the K'_fs of some tetraazamacrocyclic copper complexes.

Spectrophotometry is another technique that can be used for the determination of the K'_fs. However, the slow binding/decomplexation kinetics of the cross bridged ligands limits the efficacy of this technique. An alternative to the potentiometric and spectrophotometric methods is the use of a method based on chromatography. Evidence from several experiments in the literature supports the use of chromatographic methods for the determination of K'_fs. For example the K'_fs for polycyclic aromatic compounds, pentacyclic triterpene acids complexes with cyclodextrins and metal complexes have been measured using chromatography. [37,38] As part of the research presented here a model study was undertaken to prove that chromatography could be used to determine

the K'_fs for the complexation of tetraazamacrocyclic ligands with copper using Cu-cyclam and Cu-cyclen complexes (Figure 1.3).

1.4 Chromatographic studies of copper complexes with methane-phosphonate pendant arm ligands

Chromatographic methods have been utilized for the determination of physicochemical properties including acid dissociation constants (K_a) for a range of analytes. The K_a of oxalic acid, anilines and other amines has been determined using RP-HPLC [39,40] The K_a is a key parameter affecting important properties of an analyte including solubility, lipophilicity and membrane permeability.[40] Shen was able to successfully estimate the two K_as for the copper complex, Cu-TETA, using chromatography.[35] The values obtained by Shen using the HPLC method compared favorably to values reported in the literature measured using potentiometry.

The most common methods for measuring K_a are potentiometric and spectrophotometric titrations. Spectrophotometry is a very sensitive technique but is labor intensive and time consuming. The technique is based on measuring absorbance at two different wavelengths and requires a chromophore which is near the ionization center to allow accurate K_a values to be determined.[39] The ionized and neutral forms of the analyte are expected to have different molar absorptivities. The presence of the chromophore near the ionization center allows for a more distinguishable difference between the two different ionization states. HPLC offers some major advantages over the spectrophotometric and potentiometric techniques used for the determination of K_as. For

instance the sample does not need to be pure and only small quantities of sample are required.[41,42]

The determination of acid dissociation constants based on chromatographic methods was first reported in the mid 1970s.[44] It was then that the relationship between the capacity factor and the acid dissociation constants was reported.[44] In chromatography the partitioning of the solute between the stationary phase and the mobile phase is related to the capacity factor. The capacity factor can be evaluated from the chromatogram and is defined in Equation 1.1:

$$k' = \frac{(t_r - t_0)}{t_R} \quad \textbf{(Equation 1.1)}$$

where t_R, is the retention time for the analyte sample component under investigation and t_0 is the retention time of an unretained solute. The t_0 value can be obtained from the retention time of sodium nitrate solution in water using a mobile phase of 100% methanol to elute it form the C_{18} stationary phase. Equation 1.2 given below shows that k' for an ionizable analyte is dependent on its acid dissociation constants and the pH of the mobile phase.[44] Equation 1.2 applies when the analyte is a monoprotic acid:

$$k' = \frac{k_0}{1 + \frac{K_a}{[H^+]}} + \frac{k_{-1}}{1 + \frac{[H^+]}{K_a}} \quad \textbf{(Equation 1.2)}$$

where k_0 and k_{-1} are defined as the capacity factors for the neutral (protonated, HA) and completely ionized (conjugate base, A^-) forms of the acid, and K_a, is the ionization constant. The retention of the weak monoprotic acid on a C_{18} stationary phase will be greater in acidic solution where the weak acid is undissociated in comparison to basic solution where it will be dissociated. For a diprotic acid there is also a relationship

between the capacity factor and the respective K_as of the analyte defined in Equation 1.3. [43] Horvath derived the following relationship between the capacity factor and the acid dissociation constants for the analyte: [43]

$$k' = \frac{k_0 + k_{-1}\frac{K_{a1}}{[H^+]} + k_{-2}\frac{K_{a1}K_{a2}}{[H^+]^2}}{1 + \frac{K_{a1}}{[H^+]} + \frac{K_{a1}K_{a2}}{[H^+]^2}} \quad \textbf{(Equation 1.3)}$$

where k_0, k_{-1}, and k_{-2} are the capacity factors for the neutral (protonated, H_2A), the amphiprotic (HA^-), and completely ionized (conjugate base, A^{2-}) forms of the acids respectively. The corresponding acid dissociation constants in the mobile phase for the analyte are given by K_{a1} and K_{a2}.

No modifiers such as methanol or acetonitrile were incorporated into the mobile phase used in this work which allows the K_a values estimated using HPLC can be compared to the values measured by potentiometry or spectrophotometry. The presence of an organic modifier such as methanol would make it impossible to accurately determine the aqueous pH of the solution, only an apparent pH would be obtained. In some cases when using a spectophotometric technique to measure pK_a a buffer may be used to control the pH of the solution and affect the dissociation of the analyte being investigated. [44] Therefore in the experiments carried out to determine the two K_as for Cu-CB-TE2P (Figure 1.4) the mobile phase contained a minimal amount of buffer to control the pH. The pH of the mobile phase determines the species of Cu-CB-TE2P that will be present in solution and affects its chromatographic behavior. A plot of the capacity factor vs. pH is similar to the form of a potentiometric titration in which the pH of the solution changes as more base or acid is added to the solution.

The chromatographic behavior of Cu-CB-DO2P^{OEt}, Cu-CB-TE2P^{OEt}, and Cu-CB-TE2P on both C_{18} and PGC stationary phases is highly dependent on their lipophilicity. The structures of these complexes are presented in Figure 1.11.

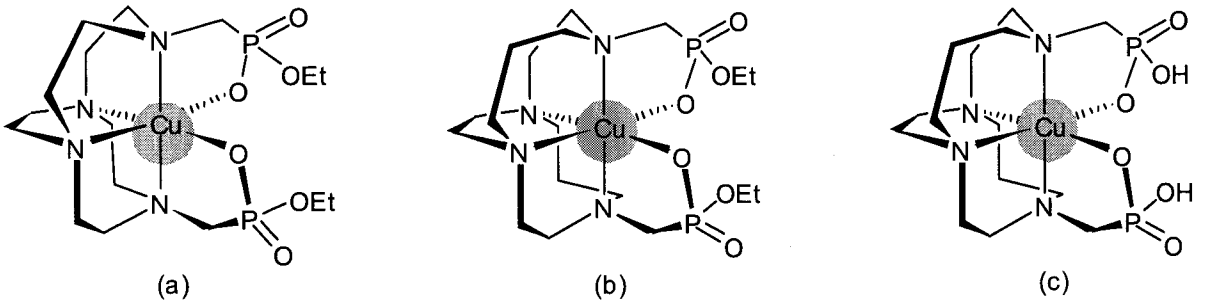


Figure 1.11. Structures of (a), Cu- CB-DO2P^{OEt}; (b), Cu-CB-TE2P^{OEt}; (c), Cu-CB-TE2P

Furthermore, lipophilicity may help to predict a complex's *in vivo* behavior and permeability through membranes.[45-47] While there are many definitions in the literature for the term "lipophilicity", the following operational definition has been recommended by IUPAC: "Lipophilicity represents the affinity of a molecule or a moiety for a lipophilic environment. Lipophilicity is commonly measured by a solute's distribution behavior in a biphasic system, either liquid-liquid (*e.g.*, partition coefficient for 1-octanol/water) or solid-liquid (retention in reversed-phase high-performance liquid chromatography (*RP-HPLC*) or thin-layer chromatography (*TLC*) system)".[47] The partition coefficient (P) is the ratio of the concentrations of the compounds in two immiscible solvents. Coefficients for solute portioning between various solvents have been studied but the 1-octanol/water partition coefficient is generally accepted as an indicator for the lipophilicity of compounds. The relative lipophilicities of drugs, metabolites and other analytes are found in the literature as log P values.[47]

RP-HPLC has been used to evaluate the lipophilicity for a wide range of analytes including copper complexes, aklylbenzenes, pesticides, phenols, and aromatic acids.[35,45,46] The HPLC method has several advantages over the "shake-flask" method often used to measure partition coefficents including speed, smaller sample size, greater sensitivity, reduced sample handling, and reproducibility. [35, 45] Another major advantage of HPLC as previously mentioned for the measurement of K_a is that it does not require a pure sample. Due to the inherent separation provided, the HPLC method is generally not affected by degradation products or impurities in the sample.

1.5 Separation of chiral cross-bridged polycyclic tetraamines including CB-cyclam, dimethyl dibenzo CB-cyclam and dibenzobisaminal

Many of the drugs marketed today are racemic mixtures. However, enantiomers may have different *in vivo* behavior including activities and toxicities.[47-50] The FDA requires pharmacological tests to adequately assess the behavior of each enantiomer.[47] Cross-bridged tetraazamacrocyclic ligands are used as chelating agents in metal complexation. The metal complexes of these ligands may potentially be used as diagnostic or therapeutic agents. Therefore developing separation conditions for the ligands would be desirable not only for evaluation of the fundamental properties of each enantiomer but also to assess chiral purity.

As the pharmaceutical industry strives to provide enantiopure drugs HPLC methods are widely used to provide chiral separations. [47-50] There are two primary approaches, indirect and direct, for resolving enantiomers using chromatography. The indirect methods involve reacting an optically active, stable and enantiomerically pure

derivatization reagent with the enantiomeric pair. The indirect approach is often more cumbersome to utilize as the derivatization of the enantiomers, which yields two diastereomers, is not always a straightforward process. The diastereomers differ from the enantiomers in that the diastereomers posses different chemical and physical properties. Diastereomers can be separated using techniques such as achiral chromatography, fractional recrystallization and distillation. The derivatization of amino acids using chloroformates, carboxylic acids, isothiocyanates, and isocyanates results in diastereomers that can be separated on reversed-phase stationary phases.[51] Some of the advantages of the indirect method include the low cost of achiral columns, potentially simpler methods development, and the possibility of enhancing detection sensitivity by using a derivatizing agent that contains a fluorophore or chromophore. Some of the disadvantages of the indirect method are the fact that the purity of the chiral derivatizing agent is critical, derivatization may be time consuming, derivatization might not go to completion, and the molar absorptivities of the diastereomers may be different from each other. Another disadvantage of the derivatization method is the possibility of side products being formed or excess reagent interfering with the separation of the diastereomers.

While for the indirect approach the enantiomers are reacted and converted to diastereomers prior to analysis by HPLC, in the direct approach no modifications to the enantiomers are done prior to injection on the column. One direct approach for obtaining a chiral separation is to add a chiral agent to the mobile phase. The interactions between the chiral agent and one or both of the enantiomers may allow a separation to be achieved when using an achiral stationary phase. Chiral resolution is based on differences in the

stabilities of the diastereomeric complexes formed in the column, solvation in the mobile phase, or binding of the complexes to the stationary phase. [47,50] Cyclodextrins are often used as chiral agents due to their structural properties. Cyclodextrins have a stereospecific doughnut-shaped structure.[47] Cyclodextrins are cyclic oligosaccharides of α-D- glucose units linked through the 1,4 position. The most common forms of the cyclodextrins are α-, β-, and γ-cyclodextrin with six, seven, and eight glucose units respectively. β-cyclodextrin has been successfully employed as a chiral mobile phase additive to achieve the separation of chiral barbiturates such as mephenytoin, methylphenobarbital, and hexobarbital.[47] A permethylated β-cyclodextrin was used as a mobile phase additive to obtain the enantiomeric separation of glutathimide and 2,2'-dihydroxy-1,1-binaphthol.[52] β-cyclodextrin, and its hydroxypropyl, methyl and sulphate derivatives were also used as chiral mobile phase additives to obtain the separation of ibuprofen enantiomers.[53]

In addition to the cyclodextrins, it is possible to use other mobile phase additives to produce chiral separations. Transition metal complexes are among the chiral resolving agents that have produced chiral separation when added to the mobile phase. The initial studies showing the viability of these chiral additives as resolving agents date back to Davankov and Rogozhin in the 1970s.[48,49] Copper (II) complexes of L-proline, L-arginine, L-histidine and L-histidine methyl ester have been used successfully to obtain enantioseparations on achiral stationary phases. [48,49] Davankov and Rogozhin used Cu (II)-proline as a chiral additive since its sterical rigidity provides a higher degree of enantioselectivity. In the case of amino acids, the D an L isomers can form diastereomeric binary or ternary complexes of different stabilities with the copper

complexes allowing for chiral resolution. The retention of the amino acids can be modified by changing the concentration of the copper complexes in the mobile phase. In general, increasing the concentration of the additive resulted in increased retention for the diastereomers of the aminoacids. [48,49]

The large number of commercially available chiral additives provides more opportunities to optimize the enantiomeric separation by changing chromatographic conditions. Chiral additives are used with achiral stationary phases, which often lowers the overall cost of the analysis. One of the disadvantages includes the possibility of more time and effort being required to develop the method. Multiple experiments may need to be conducted with various chiral additives to successfully resolve the enantiomeric pair. Another disadvantage is the possibility of the chiral additive interfering in the detection of the analyte.

Another direct chromatographic approach for obtaining a chiral separation is the use of a chiral stationary phase. Since the 1980's chiral stationary phases have become widely used due to their increased commercial availability.[49] Presently, there is a wide variation in the chiral stationary phases commercially available, ranging from chiral polymers (cellulose, amylose, cellulose triacetate) and proteins (bovine serum albumin), to bonded cyclodextrins. [3,48] The use of a chiral stationary phase tends to yield more reproducible results compared to the previously described derivatization method.[48] In addition, it is easier to obtain the desired enantiomer following the separation when using chiral stationary phases compared to using either the derivatization method or addition of chiral mobile phase additives.

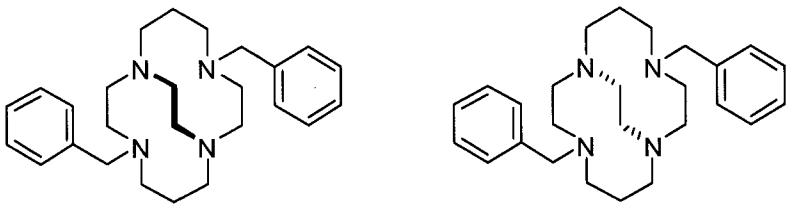
Berthod has recently published reviews discussing the separation of enantiomers for a variety of drugs. [3,49] These reviews show the increased use of chiral stationary phases and the wide range of stationary phases currently available. The most commonly used chiral stationary phases are the polysaccharide stationary phases. [3,49] The extensive use of polysaccharide based chiral stationary phases is due to their inherent advantages. These advantages include a broad spectrum of enantioselectivity, higher loading capacity, and the ability to easily recover the purified enantiomers. [3,49]

Berthod identified the most commonly used chiral stationary phases for the enantioseparation of various drugs. The drugs are segregated into thirteen classes using the Anatomical and Therapeutic Chemical classification.[3, 49] The number of drugs for each category varies, therefore, it is important to look at the number of overall separations achieved on specific stationary phases. Though the use of polysaccharide chiral stationary phases varies from class to class, they produce 38 % of the total reported enantiomeric separations. This is important because it is twice the number of the separations of the next most successful stationary phase, which are the Pirkle-based stationary phases. The most successful chiral stationary phases for each class of drugs are shown in Table 1.1. The fact that many different drug enantiomers can be baseline resolved using a polysaccharide chiral stationary phases proves their versatility. [3, 49]

Drug Classification	Most successful chiral stationary phase	% of separations	Next most successful chiral stationary phase	% of separations
1. Alimentary tract drugs	Polysaccharide	51	Protein	18
2. Anti-infectives	Polysaccharide	29	Antibiotic	18
3. Antineoplastic and immunomodulating agents	Polysaccharide	62	Antibiotic	18
4. Antiparasitic, insecticide drugs and repellents	Pirkle	49	Polysaccharide	25
5. Blood and blood-forming organs	Polysaccharide	38	Protein	19
6. Cardiovacular system	Polysaccharide	37	Pirkle	18
7. Dermatological Drugs	Polysaccharide	83	Protein	8
8. Genito-urinary system and Sex hormones	Polysaccharide	54	Cyclodextrin	24
9. Muscolo-skeletal System	Polysaccharide	36	Protein	17
10.Nervous system	Polysaccharide	41	Protein	16
11. Respiratory system	Polysaccharide	33	Pirkle	25
12. Sensory organs	Polysaccharide	44	Protein	28
13. Various drugs	Antibiotic	34	Polysaccharide	13

Table 1.1. Separation of clinical racemic drugs using commercial chiral stationary phases (based on the data provided in reviews by Berthod in his reviews) [3,49]

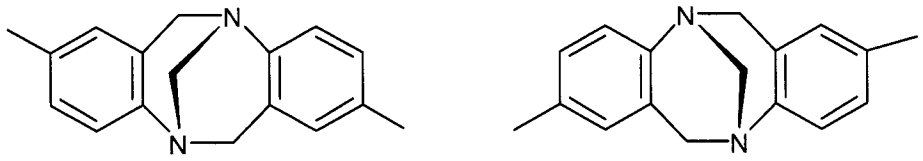
The cross-bridged ligands have chiral centers due to the ethylene bridge. A study performed on dibenzyl CB-cyclam (Figure 1.12) using NMR spectroscopy revealed the slow tucking of the bridging ethylene unit through the middle of the 14-membered ring (homeomorphic isomerization).[12]



(a) (b)

Figure 1.12. Structures of dibenzyl CB-cyclam enantiomers (a, b)

A well known compound that has a similar chiral center is Tröger's base (Figure 1.13). Tröger's base is a chiral heterocyclic amine which has chirality arising from the two stereogenic nitrogens in the macrocycle. Chromatographic resolution of the enantiomers of Troger's base, and related racemic compounds, has been reported in the literature.[55-57]



(a) (b)

Figure 1.13. Structures of Tröger's base enantiomers (a,b)

A study of the adsorption behavior of the enantiomers of Tröger's base on an amylose tris(3,5-dimethyl carbamate) stationary phase revealed that different van der Waals interactions seem to be responsible for the enantiomeric selectivity.[56] In addition, Sergeyev reported the separation of the enantiomers of Tröger's base with a resolution greater than 4 using a mobile phase of ethanol on a Chiralcel OJ stationary phase.[56] Chiralcel OJ is a cellulose tris (4-methylbenzoate) stationary phase coated on 10 μm silica gel. In an effort to better understand the selectivity of the Chiralcel OJ stationary phase Sergeyev looked at the enantioseparation of Tröger's base derivatives. The

enantioseparation for five out of the six pairs of enantiomers (Figure 1.14a) was achieved under the same conditions used for the separation of the Tröger's base enantiomers. However, for one of the derivatives (Figure1.14b), baseline resolution of the enantiomers was obtained using a mobile phase of 83/5/12 (v/v/v) n-hexane/ethyl acetate/ethanol on a Whelk O1 stationary phase. [56]

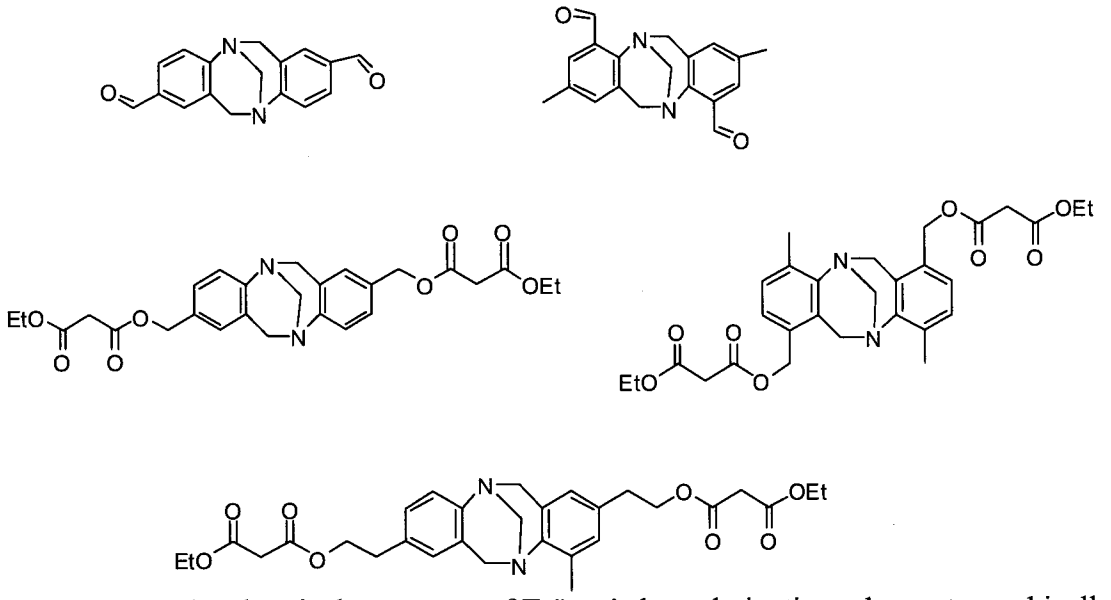


Figure 1.14a. The chemical structures of Tröger's base derivatives chromatographically separated by Sergeyev [56]

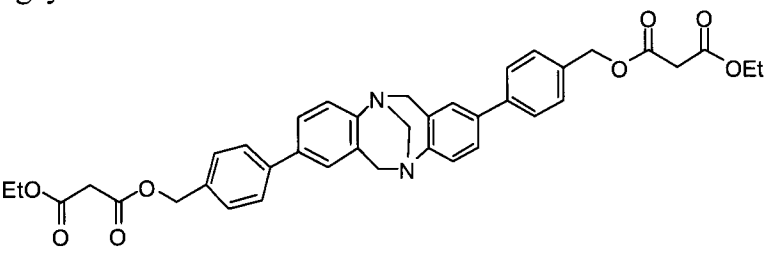


Figure 1.14b. The chemical structure of Tröger's base derivative chromatographically separated by Sergeyev [56]

The Whelk-O1 CSP is formed when 1-(3,5-dinitrobenzamido)- 1,2,3,4-tetrahydrophenanthrene is attached to a surface by a short alkyl tether and a siloxyl group. The selectivity of this stationary phase arises from the fact that the overall shape is a cleft. Didier demonstrated that Whelk O1 could be utilized as an alternative to the polysaccharide stationary phases to obtain the separation of Tröger's base enantiomers. Most of the enantiomers of the 20 derivatives of Tröger's base were resolved with 95/5 (v/v) hexane/isopropanol on the Whelk O1 CSP while others required mobile phases such of 98/2 (v/v) or 80/20 (v/v) hexane/isopropanol to be resolved. [57] The structures of selected Tröger's base derivatives successfully resolved are presented in Figure 1.15.

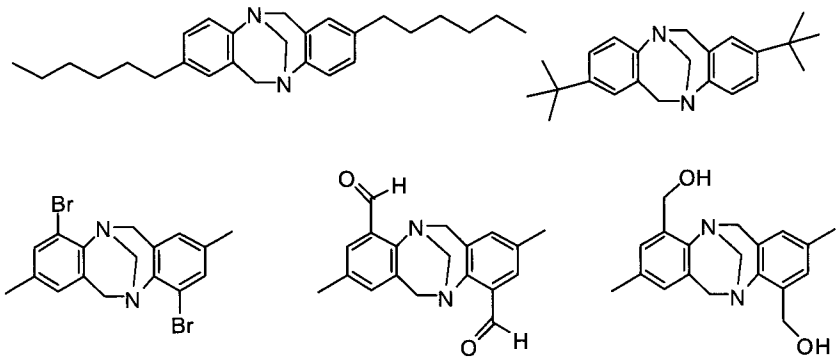


Figure 1.15 The chemical structures of Tröger's base derivatives chromatographically separated by Didier using a Whelk O1 CSP [57]

It is important to develop chiral separation conditions in order to evaluate enantiomeric purity rapidly. The presence of chromophores in the enantiomer allows evaluation of the enantiomeric purity with greater ease in comparison to techniques such as NMR. For example the enantiomeric purity of CB-cyclam (Figure 1.16) can be directly assessed using HPLC by simply diluting and injecting the sample. By comparison, to quantify the enantiomeric ratio for CB-cyclam using NMR spectroscopy

the sample needs to be converted to diastereomers which is more time consuming compared to the HPLC analysis and requires more sample.

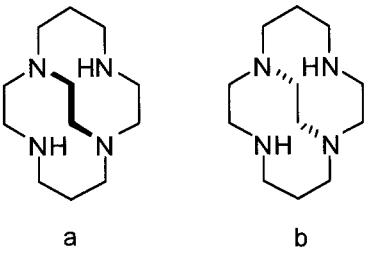


Figure 1.16. Structures of CB-cyclam enantiomers (a, b)

In addition to CB-cyclam the chromatographic behavior of the enantiomers of dibenzocyclam bisaminal and dibenzodimethyl CB-cyclam (Figure 1.17) were investigated. The analytes in this study were eluted with organic mobile phases enhancing the opportunity to scale up the separation using preparative chromatography. The pure compound can be isolated more easily after the separation when volatile organic modifiers are used.

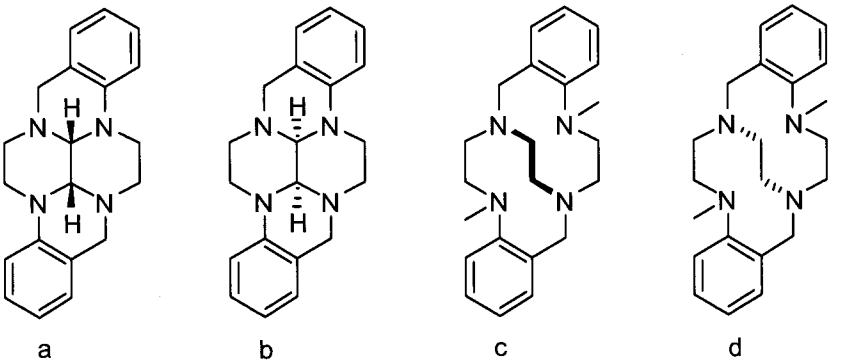


Figure 1.17 Structures of dibenzocyclam bisaminal enantiomers (a,b) and dibenzodimethyl CB-cyclam enantiomers (c,d)

Chromatographic enantiomeric separations have been used to determine the racemization barrier ($\Delta G^{\ddagger}$).[102-104] HPLC provides a fast, direct and reliable method for

determining $\Delta G^{\ddagger}$ once chiral chromatographic conditions have been established. HPLC has been used to measure $\Delta G^{\ddagger}$ for the enantiomers of many compounds including salts of 1,4,5,6 tetrahydropyrimidinium, N-(o-aryl)-2-thioxo-oxazolidine-4-one, rhodanine derivatives, and 2-arylimino-3-aryl-thiozolidine-4-one derivatives.[102-104]

NMR spectroscopy was used by Weisman and co-workers to determine the $\Delta G^{\ddagger}$ for selected ligands.[11] Hines and Weisman pointed out that if $\Delta G^{\ddagger}$ of enantiomerization is at least 25 kcal/mol at 25 °C for any ligand, their enantiomers should be stable (slow racemization) for at least a few hours. For example, if the half-lives of the ligands are approximately 66 hours at 25 °C the racemization would be considered slow. It was shown by Hines and Weisman for ligands such as dibenzyl CB-cyclam with a $\Delta G^{\ddagger}_{344K}$ of 28 kcal/mol, that the enantiomers can theoritcally be resolved at room temperature. The high racemization barrier means that enantiomerically pure dibenzyl CB-cyclam will not racemize quickly at room temperature.

Odendaal determined $\Delta G^{\ddagger}$ of racemization for CB-cyclam utilizing NMR spectroscopy. [11] A mixture of CB-cyclam enantiomers in solution was converted to diastereomers using enantiomerically pure L-(+)-tartaric acid. The two diastereomers exhibit different proton shifts allowing for determination of enantiomeric excess by measuring integrated peak heights. Odendaal and Weisman determined a $\Delta G^{\ddagger}_{355\,K}$ of 31.29 ± 0.10 kcal/mol for the enantiomers of CB-cyclam.[11]

In addition to NMR spectroscopy, HPLC can be used to determine the enantiomerization barrier of cross-bridged tetraazamacrocyclic ligands. One of the goals of this research was to determine $\Delta G^{\ddagger}$ of CB-cyclam utilizing HPLC conditions after establishing the separation of the CB-cyclam enantiomers on a chiral stationary phase.

HPLC could be an alternative to NMR for evaluation of enantiomeric barriers of other tetraazamacrocyclic ligands.

CHAPTER II

EVALUATION OF CHROMATOGRAPHIC PROPERTIES OF SELECTED COPPER COMPLEXES WITH CROSS-BRIDGED TETRAAZAMACROCYCLIC LIGANDS AND DETERMINATION OF CONDITIONAL FORMATION CONSTANTS

2.1 Introduction

Knowledge of the stability of the copper metal complexes with cross-bridged tetraazamacrocycles is important for possible *in vivo* applications. Chromatographic techniques have been used to determine physico-chemical properties such as conditional formation constants (K'_f), acid dissociation constants and lipophilicity or hydrophobicity parameters. [58-61] One of the goals of this research was to investigate the use of HPLC for obtaining thermodynamic stability data for copper cross-bridged complexes. It is of particular interest to establish the K'_fs for the following complexes: Cu-CB-cyclam, Cu-CB-TE2A and Cu-CB-TE1A in order to compare them to Cu-cyclam and Cu-cyclen (Figures 1.2 and 1.3). The comparison of the K'_fs for the complexes mentioned above will allow the establishment of thermodynamic trends based on macrocycle size, pendant arm length and type.

In order to determine the K'_fs for selected copper complexes using chromatographic methods, separation conditions for these metal complexes were established. Separations for the complexes of interest, including Cu-CB-TE2A,

Cu-CB-TE1A, Cu-CB-cyclam, Cu-cyclam, Cu-CB-TEAMA, Cu-CB-cyclen, and Cu-cyclen (Figures 1.2 and 1.3) were investigated under both ion exchange and reversed phase conditions. All of the above complexes except Cu-CB-TE2A are cations below pH 8, which is why the use of ion-exchange chromatography was initially investigated. A strong cation exchange stationary phase was utilized to investigate the separation of the analytes under different mobile phase conditions. The effect of several cations (Ca^{2+}, Na^{+} and NH_4^{+}) at different concentrations on the chromatographic behavior of the complexes when using a strong cation-exchange stationary phase was studied. In addition the effects of the organic modifier (acetonitrile and methanol) and temperature on the retention times for selected copper complexes was evaluated.

Ion exchange chromatography (IEC) is typically used for the separation of charged or easily ionized analytes. IEC finds applications in all areas of chemistry.[62-64] Most importantly it has been utilized for the separation of amino acids, peptides, proteins, nucleotides and biopolymers.[62] In addition IEC has been used for analysis of carbohydrates, organic and inorganic ions. [62-64] The major application of IEC remains the separation of inorganic anions and organic cations in industrial, agricultural, food and environmental samples. Most of the metals in the periodic table have been separated using IEC at one time or another.[63]

In IEC the main factor leading to the retention of the analytes is the affinity of the analyte and the mobile phase ions for the immobilized counterions of the stationary phase.[63] The analyte ions and eluent ions interact with multiple stationary phase ion centers as they pass through the column. The analyte ions are separated based on

differences in their relative affinity for the stationary phase ion centers compared to those of the mobile phase in a dynamic exchange system.

In addition to using a strong cation exchange stationary phase, the use of a porous graphitic carbon (PGC) stationary phase was also investigated. It has been demonstrated that charged analytes can be separated on the PGC stationary phase. PGC is another type of reversed stationary phase manufactured originally by the method of Knox and Gilbert.[65] It offers several advantages over the alkyl bonded silica stationary phases. For example, PGC is not limited by the hydrolytic stability of silica-based packing materials. [65] Furthermore, PGC is free of impurities whereas the silica based material may contain unreacted silanols and a variable content of metallic impurities.

PGC is a crystalline and highly reproducible material which makes it ideal for use as a stationary phase.[65] PGC stationary phase have demonstrated good mechanical and chemical stability, surface homogeneity, and surface area, and particle size distribution.[65] The chromatographic behavior of the analytes on the PGC stationary phase arises from its unique properties. It was first marketed under the Hypercarb name in 1988 by Shandon HPLC and is now marketed by Thermo Scientific.[66] Since PGC's introduction as a stationary phase there have been many applications. PGC can be classified as an adsorbent where the carbon surface acts as a Lewis base towards polar solutes and is involved in π-π interactions and dispersive interactions with aromatic solutes. [66] Retention was found to increase with increasing numbers of polar substituents for the analyte.[66] In addition in the same study the authors found that retention was shown to depend on the position of substituents on the aromatic ring.[68] The chromatographic

performance of the PGC stationary phase has improved, comparing favorably in terms of peak symmetry and theoretical plate height to silica bonded stationary phases.

Despite the advances, PGC represents an underutilized stationary phase for separating lipophilic compounds by RPLC. PGC has shown potential for the discrimination of lipid species containing carbon double bonds and glycolipids. [67] Interactions between the carbon double bond and the surface of the PGC stationary phase is expected due to its polarizable nature. The number of double bonds and the conformation of the molecule are two additional factors that affect the chromatographic behavior of the analytes. [67] In the few studies employing non-aqueous mobile phases with PGC, increasing in the hydrocarbon chain length of the solute always resulted in an increase in retention, as would be expected under reversed phase elution conditions. PGC demonstrates increased selectivity and the ability to resolve geometrical isomers which can not be resolved using alkyl bonded silica or polymer based stationary phases. [66]

Lim and coworkers, observed peculiar behavior of the PGC stationary phase when compared to the C_{18} silica bonded stationary phases.[68] In their work studying the separation of pertechnatate and perrhenate anions on PGC with predominantly aqueous eluents, separations based on additional interactions besides hydrophobic interactions were observed. [68] The ability to separate charged complexes arises from the properties of the graphite surface. Their data supported an interaction between the charged centers of the analyte and the graphite surface. The mechanism could not simply be an ion exchange mechanism as cations and anions were separated in a single run. In addition to Lim there are other reports in which the PGC stationary phase has been used for the separation of charged complexes.[65]

The viability of HPLC as a technique for measuring the K'_f for copper tetraazamacrocyclic complexes will be tested using a model study. The K'_f for Cu-cyclen was determined and compared to the literature value. The two complexes of the model study, Cu-cyclen and Cu-cyclam, are closely related to the copper complexes of primary interest, including Cu-CB-cyclam and Cu-CB-TE2A. (Figures 1.2 and 1.3). Both Cu-cyclen and Cu-cyclam have perchlorate as the counterion. The perchlorate counterions do not interfere in the analysis of the K'_fs. In solution, these complexes dissociate from the counterion and the metal complex has a +2 charge. The difference between the two complexes used in the model study is ring size, shape and binding conformation. Cu-cyclam has two more methylenes in the ring than Cu-cyclen. In order to determine the K'_fs two solutions were prepared. The first solution was Cu-cyclam and cyclen and as it approached equilibrium Cu-cyclen and cyclam were formed as shown in Equation 2.1a. The second solution was Cu-cyclen and cyclam and as it approached equilibrium Cu-cyclam and cyclen were formed as shown in Equation 2.1b. Equilibrium is reached once there is not a significant change in the concentrations of Cu-cyclam and Cu-cyclen. Depending on the pH of the solution, the value of the relative conditional formation constants will vary based on the degree of ionization of the ligands. For the solutions shown in Equation 2.1 the pH was held at 10.5.

$$\text{Cu-cyclen} \rightleftharpoons \text{Cu-cyclam} \quad \textbf{(Equation 2.1a)}$$

$$\text{Cu-cyclam} \rightleftharpoons \text{Cu-cyclen} \quad \textbf{(Equation 2.1b)}$$

Equations 2.2 and 2.3 show the formation constants for the Cu-cyclen and

Cu-cyclam complexes respectively. The values for the K'fs of both complexes can be calculated since the absolute formation constants and ligand pK_a's are available in the literature.[69,70] Equation 2.4 shows the competition reaction taking place between Cu-cyclen and cyclam. At equilibrium there will be a mixture of both complexes Cu-cyclen and Cu-cyclam as well as the free ligands cyclam and cyclen.

$$K_{1Cu-cyclen} = \frac{[\text{Cu - cyclen}]}{[Cu^{2+}][\text{cyclen}]} \quad \textbf{(Equation 2.2)}$$

$$K_{2Cu-cyclam} = \frac{[\text{Cu - cyclam}]}{[Cu^{2+}][\text{cyclam}]} \quad \textbf{(Equation 2.3)}$$

$$\text{Cu-cyclen + cyclam} \rightleftharpoons \text{Cu-cyclam + cyclen} \quad \textbf{(Equation 2.4)}$$

$$K_3 = \frac{[\text{Cu - cyclam}][\text{cyclen}]}{[\text{Cu - cyclen}][\text{cyclam}]} \quad \textbf{(Equation 2.5)}$$

$$K_{1Cu-cyclen} = \frac{K_{2Cu-cyclam}}{K_3} \quad \textbf{(Equation 2.6)}$$

K_3 will be determined experimentally (Equation 2.5), then the K'_f for Cu-cyclen will be determined as shown in Equation 2.6. The determined $K'_{1Cu\text{-}cyclen}$, will then be compared to the theoretical value. The theoretical value was calculated using the absolute formation constant (1.58×10^{27}) and adjusted to take into account the effect of pH and temperature.[69,70]

The concentration of Cu-cyclam and Cu-cyclen will be calculated from the chromatograms using the peak height method while the concentration of cyclam and cyclen will be calculated using the ligand mass balance. The analyte concentration is proportional to the peak height.[71] The working curve method was used to determine the

concentration of Cu-cyclam and Cu-cyclen. A set of standards was prepared whose concentration range brackets the initial and expected equilibrium concentration of the analyte to construct the working curve. Mass balance equations for copper ion and the ligands are shown in Equations 2.7-2.9.

$$C_{Cu\ total} = C_{Cu\text{-}cyclam} + C_{Cu\text{-}cyclen} + C_{Cu\ FREE} \quad \textbf{(Equations 2.7)}$$

$$C_{cyclam} = C_{Free\ cyclam} + C_{Cu\text{-}cyclam} \textbf{(Equations 2.8)}$$

$$C_{cyclen} = C_{Free\ cyclen} + C_{Cu\text{-}cyclen} \quad \textbf{(Equations 2.9)}$$

The amount of free copper ($C_{Cu\ FREE}$) is very small compared to the amount bound to either of the ligands, therefore it is assumed to be zero for these calculations. The concentration of the free ligands in the mass balance equations includes all protonation states of cyclam and cyclen in Equations 2.8 and 2.9 respectively.

The K'$_f$s for the Cu-cyclen and Cu-cyclam complexes were calculated using Equations 2.11 and 2.12. Equation 2.10 was used to determine the fraction of ligand (α) that will be dissociated at a given pH. The K values are the corresponding dissociation constants and [H^+] is the hydronium ion concentration. The pK_as used to calculate the α values for cyclam and cyclen are shown in Table 2.1. [70]

$$K_1 = 10^{-pKa1};\ K_2 = 10^{-pKa2};\ K_3 = 10^{-pKa3};\ \text{and}\ K_4 = 10^{-pKa4}$$

$$\alpha_{cyclam} = \frac{K_1K_2K_3K_4}{[H^+]^4 + [H^+]^3K_1 + [H^+]^2K_1K_2 + [H^+]K_1K_2K_3 + K_1K_2K_3K_4} \quad \textbf{(Equation 2.10)}$$

$$K'_{Cu\text{-}cyclam} = \alpha_{cyclam} \times K_{cyclam} \textbf{(Equation 2.11)}$$

$$K'_{Cu\text{-}cyclen} = \alpha_{cyclen} \times K_{cyclen} \textbf{(Equation 2.12)}$$

pK_a	**Cyclam**	**Cyclen**
pK_{a1}	0.80	0.80
pK_{a2}	1.50	1.60
pK_{a3}	10.30	9.49
pK_{a4}	11.23	10.51

Table 2.1. pK_a values for cyclam and cyclen[70]

In addition to the Cu-cyclam and Cu-cyclen complexes investigated in the model study, other competition reactions were set up. The determination of K'_f for Cu-CB-cyclam (Equation 2.13) was attempted by preparing a solution of Cu-CB-cyclam and cyclam and heating it to achieve equilibrium. The process of reaching the equilibrium for Equation 2.13 was slow even at 95 °C so a microwave reactor was used to bring the reaction mixture near equilibrium.

cyclam + Cu-CB-cyclam $\rightleftharpoons$ Cu-cyclam + CB-cyclam **(Equation 2.13)**

Other competition reactions with copper cross-bridged complexes such as Cu-CB-TE2A and Cu-CB-TE1A with Cu-cyclam were investigated.

2.2 Experimental

2.2.1 Reagents

ACS certified calcium chloride, reagent grade potassium acetate and sodium chloride, ACS grade sodium hydroxide, hydrochloric acid, and ammonium chloride were purchased from Fisher (Fairlawn, NJ, USA). HPLC-grade methanol was purchased from Pharmco (Brookfield, NJ, USA). HPLC-grade acetonitrile was purchased from EMD

(Gibbstown, NJ, USA). ACS grade acetic acid was purchased from EMD (Gibbstown, NJ, USA). The DI water used for the preparation of the standards and the eluents was obtained from a Milli-Q Water System. All mobile phases were filtered through a 0.45 μm nylon filter (Whatman, OR, USA) prior to use.

2.2.2 Apparatus

Chromatographic separations were performed using a Varian 9010 pump (Palo Alto, CA, USA) fitted with a Rheodyne 7125 injector (Cotati, CA, USA) having a 10 μL injection loop. UV detection was performed using a Waters 486E UV-Vis absorbance detector (Milford, MA, USA) with the wavelength set at 280 nm. The detector was interfaced to a Kipp and Zonen BD41 chart recorder. The retention properties of the copper complexes were investigated using four HPLC stationary phases: (1) Partisil 10SCX (250 × 4.6 mm; 10μm; Whatman, OR, USA); (2) Shiseido SCX (250 × 4.6 mm; 10μm; Shiseido, Torrence, CA, USA); (3) [Hypercarb] Porous Graphitic Carbon (100 × 3.0 mm; 5.0μm; Thermo Scientific, Waltham, MA, USA); and (4) Betabasic C_{18} (150 × 4.6 mm; 5μm; Thermo Scientific, Waltham, MA, USA). A Vernier pH probe (Beaverton, OR, USA) was used during the preparation of the buffer and for measuring the pH of the mobile phase.

2.2.3 Chromatographic Conditions

The mobile phase for each experiment was prepared by diluting stock solutions of the buffer in volumetric flasks. Graduated cylinders were used in the preparation of the mobile phases for the ion exchange experiments. Prior to use; each of the

chromatographic columns was equilibrated for at least 30 column volumes with the mobile phase to be used for the experiment. The ion exchange columns were flushed with 1.0 M buffer solution for 30 column volumes followed by a flush with water for another 30 column volumes. The PGC and Betabasic C_{18} column were flushed with at least 30 column volumes of 70/30 methanol/water (v/v) solution after daily use.

2.2.4 Sample Preparation

Cu(II) cyclen, Cu(II) cyclam Cu(II)-CB-cyclen, Cu(II)-CB-cyclam, Cu(II)-CB-TE2A, Cu(II)-CB-TE1A, Cu-CB-TEAMA, and Cu-TETA, were provided by the Weisman-Wong research group and synthesized according to the published methods.[10-12] Solutions of the complexes for analysis by HPLC were prepared in deionized water.

2.3 Results and Discussion

2.3.1 Chromatographic behavior of the copper (II) complexes on the strong cation exchange stationary phase

The Cu-cyclam and Cu-cyclen are cations and their chromatographic behavior was initially studied on a Partisil strong cation exchange stationary phase. The peak shape of both complexes showed tailing. The samples were diluted and even at low concentrations tailing of the peaks was present. In order to reduce tailing an organic modifier was added to the mobile phase. It has been shown that there are hydrophobic as well as ion exchange interactions on the strong cation exchange stationary phase.[62] The addition of the organic modifier will reduce the interactions between the stationary phase and the analyte providing quicker mass transfer resulting in reduced tailing. The initial

separation conditions used 1.0 M potassium ion with no organic modifier. The addition of methanol to the mobile phase improved the peak shape. A chromatogram obtained using a mobile phase composition 10/90 (v/v) methanol/1.0 M K^+ is shown in Figure 2.1.

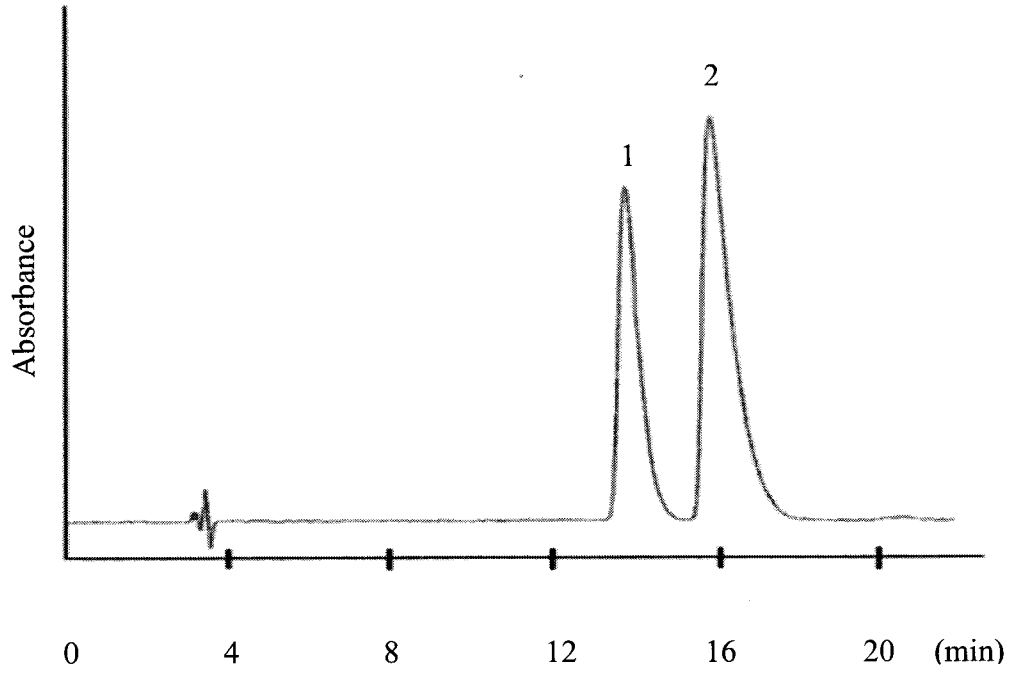


Figure 2.1. Chromatogram of (1), Cu-cyclen; (2), Cu-cyclam injected at a concentration of 0.10 mg/mL
Mobile Phase: 10/90 (v/v) methanol/1 M potassium acetate pH 5.0
Flow rate: 1.0 mL/min Column: Partisil 10SCX (250 × 4.6 mm; 10μm)
Detection λ: 530 nm Temperature: 30 °C Injection: 10 μL

Various mobile phase compositions were tried with methanol concentrations varying from 10 to 35 percent. The analysis time could be reduced but resolution between the two complexes was lost. In addition acetonitrile was investigated as a mobile phase additive. Further optimization of the separation conditions was not possible due to the lack of selectivity of the Partisil stationary phase for these two complexes. In the representative chromatograms shown in Figures 2.2.a and b, the loss of resolution between Cu-cyclam and Cu-cyclen due to high organic content can be observed.

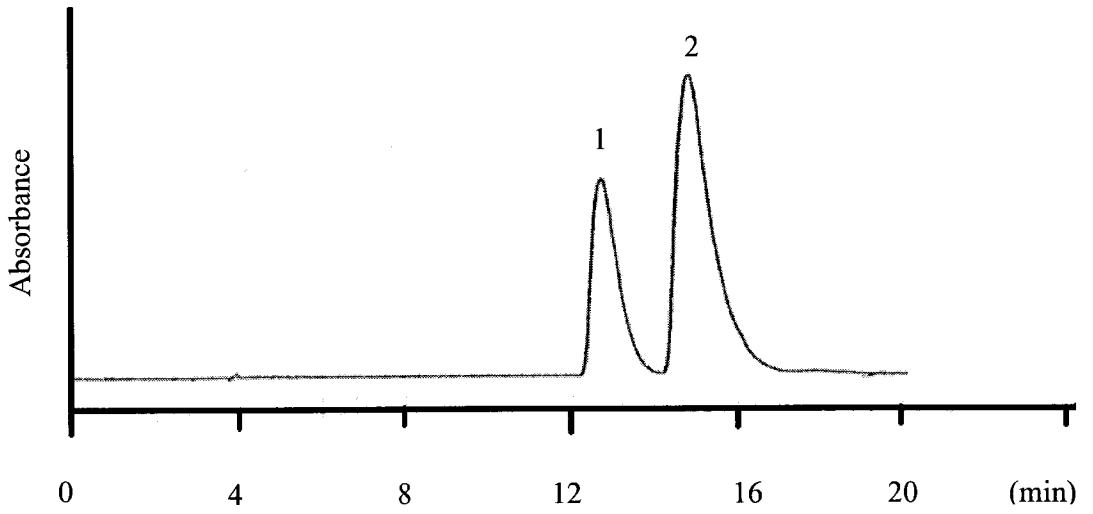


Figure 2.2.a. Chromatogram of (1), Cu-cyclen; (2), Cu-cyclam injected at a concentration of 0.10 mg/mL
Mobile Phase: 15/85 (v/v) Acetonitrile/1 M potassium acetate pH 5.0
Flow rate: 1.0 mL/min Column: Partisil 10SCX (250 × 4.6 mm; 10μm)
Detection λ: 530 nm Temperature: 30 °C Injection: 10 μL

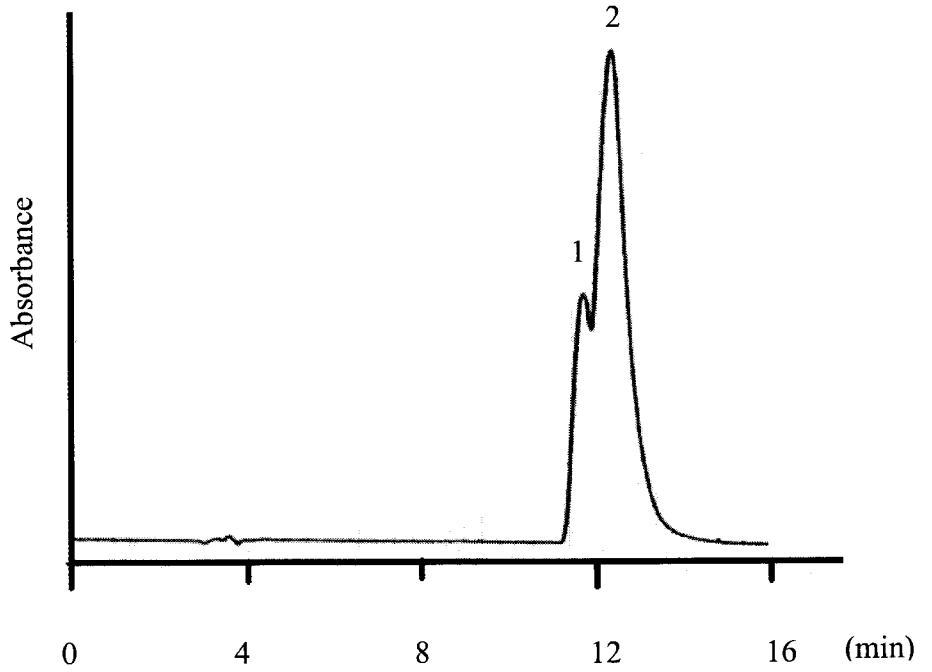


Figure 2.2.b. Chromatogram of (1), Cu-cyclen; (2), Cu-cyclam injected at a concentration of 0.10 mg/mL
Mobile Phase: 20/80 (v/v) Acetonitrile/1 M potassium acetate pH 5.0
Flow rate: 1.0 mL/min Column: Partisil 10SCX (250 × 4.6 mm; 10μm)
Detection λ: 530 nm Temperature: 30 °C Injection: 10 μL

In an effort to improve the resolution and enhance selectivity for Cu-cyclam and Cu-cyclen a Shiseido strong cation exchange stationary phase was evaluated. The analysis on the Shiseido column was performed with higher organic modifier content which produced a resolution greater than 2 for the complexes. A representative chromatogram showing the separation of Cu-cyclen and Cu-cyclam is shown in Figure 2.3.

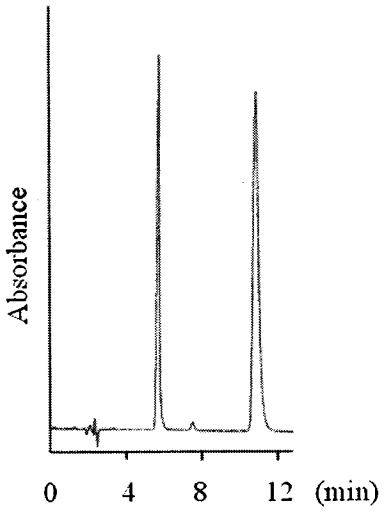


Figure 2.3. Chromatogram of (1), Cu-cyclen; (2), Cu-cyclam injected at a concentration of 0.10 mg/mL
Mobile Phase: 38/62 (v/v) Acetonitrile/ 1.0 M K^+
Flow rate: 1.2 mL/min Column: Shiseido SCX (250 × 4.6 mm; 10μm)
Detection λ: 530 nm Temperature: 45 °C Injection: 10 μL

Due to the selectivity that the Shiseido stationary phase provides for the Cu-cyclam and Cu-cyclen complexes, the separations for other copper cross bridged complexes: Cu-CB-cyclam, Cu-CB-cyclen, Cu-CB-TE1A, Cu-CB-TEAMA and Cu-CB-TE2A were investigated using this stationary phase. The effect of the added cation to the mobile phase on the chromatographic behavior of the analytes was evaluated. Four different cations: ammonium (NH_4^+), potassium (K^+), sodium (Na^+), and calcium (Ca^{2+})

were added to the mobile phase at different concentrations. The effect the first cation, NH_4^+, has on the retention of Cu-cyclam^{2+}, Cu-cyclen^{2+}, Cu-CB-cyclam^{2+}, Cu-CB-cyclen^{2+}, Cu-CB-TE1A$^+$, Cu-CB-TEAMA$^+$ and Cu-CB-TE2A (neutral) is shown in Figure 2.4. As expected a reduction in the concentration of the NH_4^+ lead to increased retention for all copper (II) complexes. The most strongly retained compound is Cu-cyclam and the least strongly retained compound is Cu-CB-TEAMA.

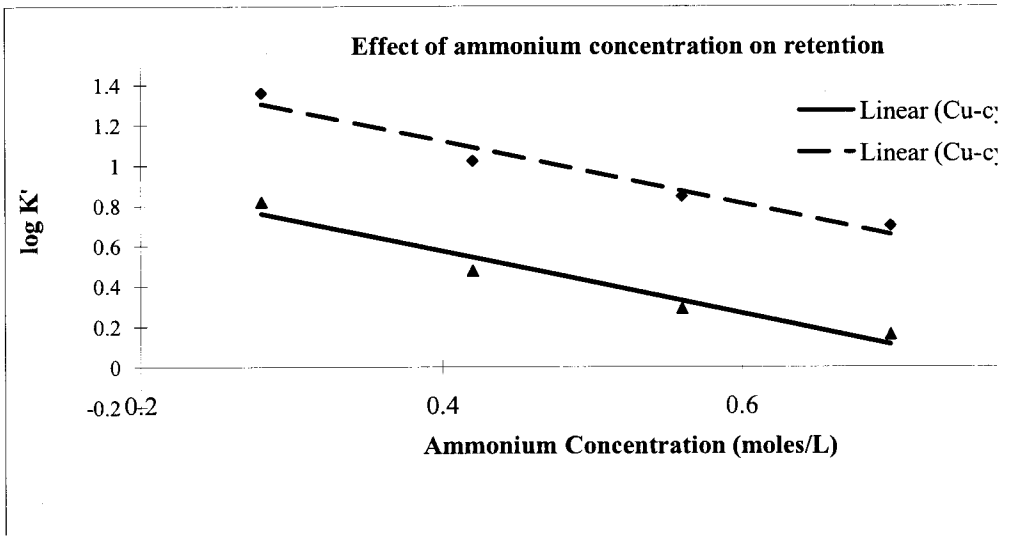


Figure 2.4a. Effect of NH_4^+ concentration on the retention of copper (II) complexes
Mobile Phase: Specified NH_4^+ concentration
Flow rate: 1.2 mL/min Column: Shiseido SCX (250 × 4.6 mm; 10μm)
Detection λ: 280 nm Temperature: 45 °C Injection: 10 μL

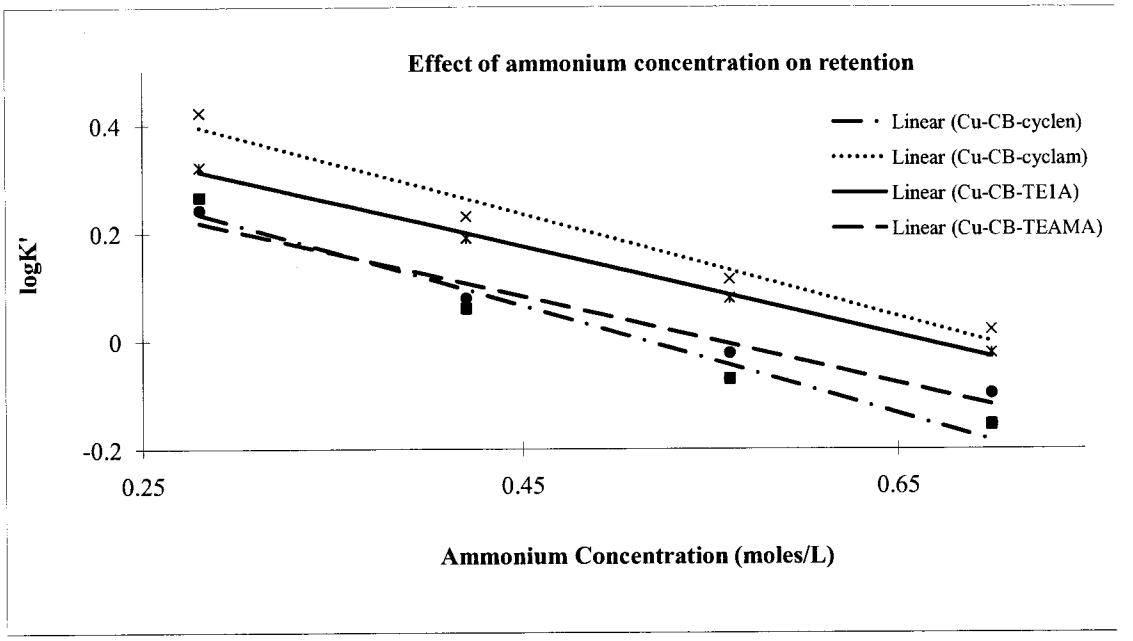


Figure 2.4b. Effect of NH_4^+ concentration on the retention of copper (II) complexes
Mobile Phase: Specified NH_4^+ concentration
Flow rate: 1.2 mL/min Column: Shiseido SCX (250 × 4.6 mm; 10μm)
Detection λ: 280 nm Temperature: 45 °C Injection: 10 μL

The other +1 charged cations, K^+ and Na^+, had a similar effect to ammonium on the retention of the six copper complexes being studied. The last cation evaluated for the separation of the complexes was Ca^{2+}. Ca^{2+} has the same charge (+2) as Cu-cyclam, Cu-cyclen, Cu-CB-cyclam, Cu-CB-cyclen. As expected in IEC a cation with a larger charge affords for the separation to be completed in a shorter amount of time. Furthermore the amount of Ca^{2+} needed to elute the complexes in similar retention times to those of the +1 charged cations (K^+, Na^+ and NH_4^+) is almost cut in half.

The presence of organic modifier in the mobile phase resulted in improved peak shape as expected and also reduced the analysis time. Various percentages of organic modifier in the mobile phase were used to elucidate the chromatographic behavior of the copper(II) complexes on the Shiseido stationary phase. A completely organic mobile

phase, 100% methanol, was investigated for eluting Cu-cyclam; however no peaks corresponding to Cu-cyclam were observed for 120 minutes. Cations in the mobile phase were required to elute the copper complexes from the Shiseido stationary phase. Results showed that for mobile phases containing cations increases in the amount of organic modifier present in the mobile phase resulted in a decrease in retention times for Cu-cyclam, Cu-cyclen, Cu-CB-cyclam, Cu-CB-cyclen, Cu-CB-TE1A, Cu-CB-TEAMA and Cu-CB-TE2A. This type of retention behavior is observed in reversed phase chromatography in which hydrophobic interactions are the most important factor in determining the elution order.

2.3.2 Evaluating the PGC stationary phase for separation of neutral and charged copper (II) complexes

Both Cu-TETA and Cu-CB-TE2A, which are neutral copper complexes at a pH below 2.6, have been previously separated on a C_{18} stationary phase by Shen.[33] In addition, Shen has reported increased retention of the copper tetraazamacrocyclic complexes on PGC under reversed phase conditions using methanol/citrate buffer as the eluent.[33] In this study, the capacity factor for Cu-TETA was approximately 4.3 on the PGC stationary phase when using a 25/75 (v/v) methanol/30mM citric acid buffer (pH ~ 2.4) as the eluent. A representative chromatogram of the elution of Cu-TETA from the PGC stationary phase is given in Figure 2.5. An impurity peak elutes around 4.2 minutes using the chromatographic conditions shown in Figure 2.5.

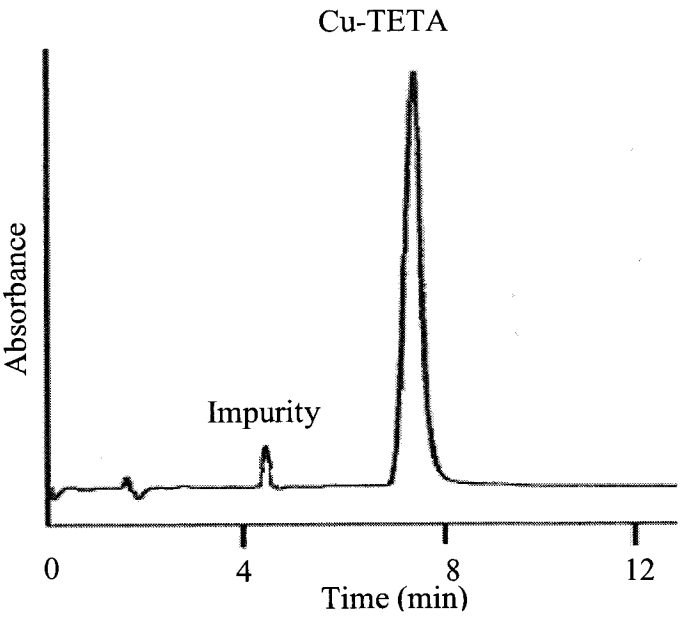


Figure 2.5. Chromatogram of Cu-TETA sample showing an impurity peak.
Mobile phase: 25/75 (v/v) methanol/30 mM citric buffer (pH ~ 2.4)
Flow rate: 0.4 mL/min Column: Hypercarb stationary phase (100 × 3mm; 5μm)
Detection: λ = 280nm Temperature: Ambient.

A sample containing $Cu(ClO_4)_2$ dissolved in water was injected and a peak appeared around the same retention time, 4.2 minutes, as the impurity. The data suggest that the impurity is either aqueous Cu(II) ions or another copper(II) salt. Cu-CB-TE2A which is another neutral copper complex has a capacity factor of 1.7 when using a 25/75 (v/v) methanol/30mM citric acid buffer (pH ~ 2.4) as the eluent. The percentage of methanol in the mobile phase was adjusted over the range of 10% to 40% organic modifier by volume and both Cu-CB-TE2A and Cu-TETA exhibited typical reverse phase behavior. A mixture of Cu-CB-TE2A and Cu-TETA was injected on the PGC stationary phase resulting in the complexes having a resolution greater than 2 using the chromatographic conditions shown in Figure 2.6.

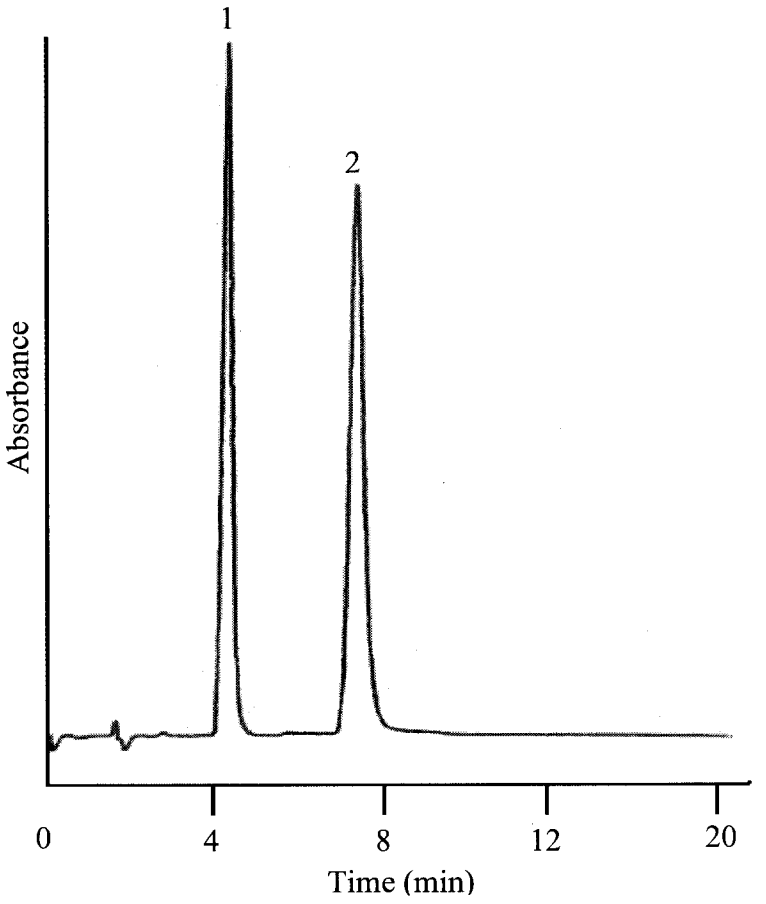


Figure 2.6. Chromatogram showing the separation of (1), Cu-CB-TE2A; (2), Cu-TETA.
Mobile phase: 25/75 (v/v) methanol/30 mM citric buffer (pH ~ 2.4)
Flow rate: 0.4 mL/min Column: Hypercarb (100 × 3mm; 5μ)
Detection λ: 280nm Temperature: Ambient

In addition to the neutral compounds, separation of positively-charged copper (II) complexes including Cu-CB-TE1A and Cu-CB-TEAMA were investigated on the PGC stationary phase. Both charged complexes, Cu-CB-TE1A and Cu-CB-TEAMA, were found to be retained on the PGC stationary phase, however no selectivity toward this pair of complexes was observed. On the other hand these copper complexes were well resolved (R_s >2) from the neutral compound Cu-CB-TE2A. The resolution of Cu-CB-

TE2A from Cu-CB-TE1A and Cu-CB-TEAMA was maintained for mobile phases ranging from 10/90 (v/v) methanol/ 30 mM citric acid (pH 2.6) to 20/80 (v/v) methanol/ 30 mM citric acid (pH 2.6) were used. Representative chromatograms showing the resolution of Cu-CB-TE2A from Cu-CB-TE1A and Cu-CB-TE2A from Cu-CB-TEAMA are shown in Figures 2.7 and 2.8 respectively.

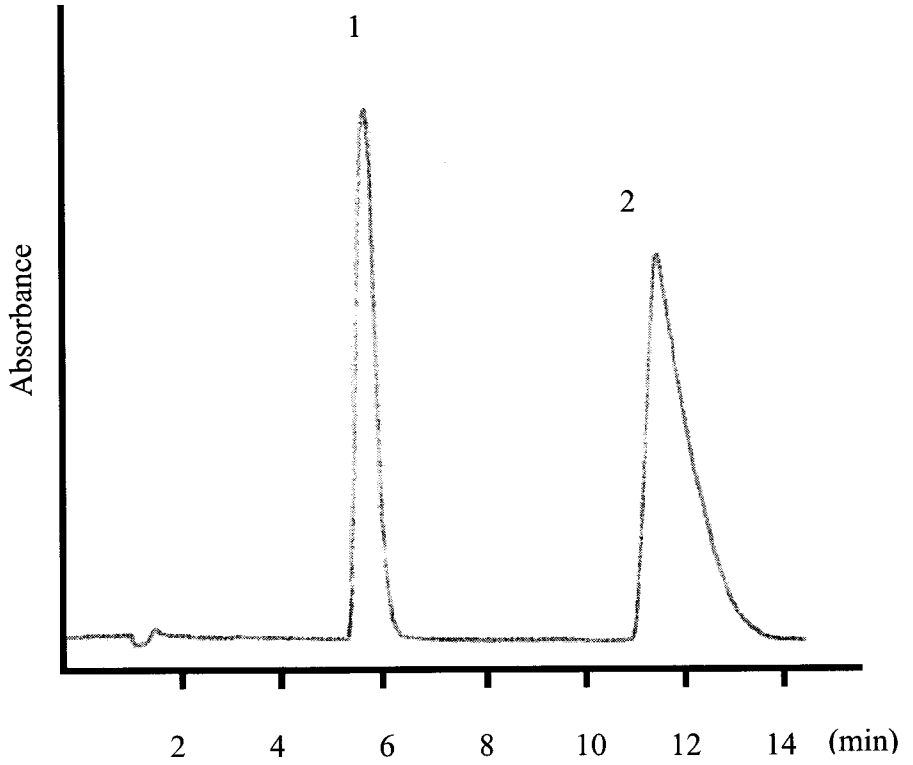


Figure 2.7. Chromatogram showing the separation of (1), Cu-CB-TE2A; (2), Cu-CB-TEAMA
Mobile Phase: 17.5/82.5 (v/v) methanol/ 30 mM citric acid pH 2.6
Flow rate: 0.4 mL/min Column: Hypercarb (100 × 3.0 mm; 5μm)
Detection λ: 280 nm Temperature: 25 °C Injection: 10 μL

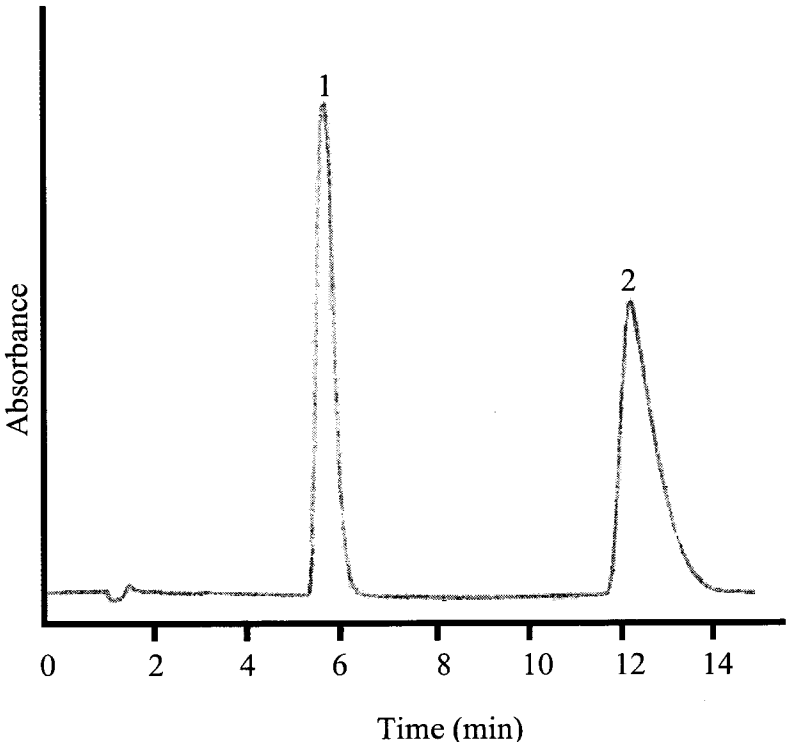


Figure 2.8. Chromatogram showing the separation of (1), Cu-CB-TE2A; (2), Cu-CB-TE1A
Mobile Phase: 17.5/82.5 (v/v) methanol/ 30 mM citric acid pH 2.6
Flow rate: 0.4 mL/min Column: Hypercarb (100 × 3.0 mm; 5μm)
Detection λ: 280 nm Temperature: 25 °C Injection: 10 μL.

Based on the chromatographic results obtained it is clear that PGC provides an alternative to strong cation exchange for the separation of the charged copper complexes. In addition, the retention of neutral copper complexes, such as Cu-CB-TE2A can be adjusted on the PGC stationary phase. It was observed that slight changes in methanol content of the mobile phase caused a significant shift in the retention times of Cu-CB-TE2A when the PGC column was used. Two other charged complexes Cu-cyclam and Cu-cyclen were evaluated on the PGC column. Even at high methanol

concentrations these compounds exhibited peak tailing. Representative chromatograms of Cu-cyclam and Cu-cyclen are shown in Figure 2.9.

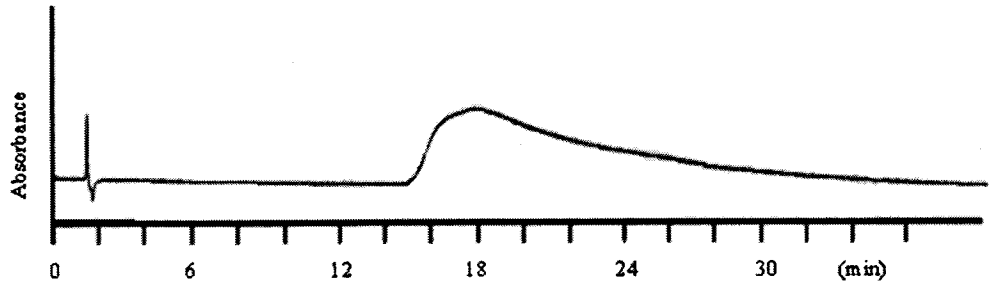


Figure 2.9a. Chromatogram showing the retention of Cu-cyclam on the PGC stationary phase
Mobile Phase: 50/50 (v/v) methanol/ 30 mM acetate buffer pH 5.0
Flow rate: 0.4 mL/min Column: Hypercarb (100 × 3.0 mm; 5μm)
Detection λ: 280 nm Temperature: 25 °C Injection: 10 μL

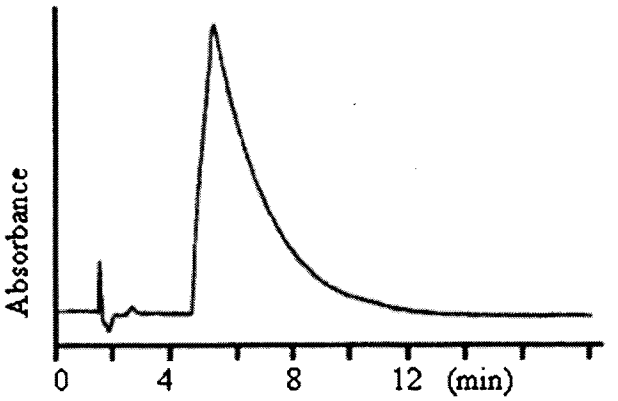


Figure 2.9b. Chromatogram showing the retention of Cu-cyclen on the PGC stationary phase
Mobile Phase: 50/50 (v/v) methanol/ 30 mM acetate buffer pH 5.0
Flow rate: 0.4 mL/min Column: Hypercarb (100 × 3.0 mm; 5μm)
Detection λ: 280 nm Temperature: 25 °C Injection: 10 μL

2.3.3 Competitive ligand binding

2.3.3a Determination of the conditional formation constant for Cu-cyclen

The model study for the determination of the conditional formation constant through a competition reaction was conducted utilizing Whatman Partisil 10SCX (250 × 4.6 mm, 10μm particle size). Various chromatographic conditions were evaluated to achieve the optimal separation conditions for Cu-cyclam and Cu-cyclen. The best chromatographic conditions, at which the analysis of the equilibrium mixture containing Cu-cyclam and Cu-cyclen was performed, utilized a 10/90 (v/v) methanol/ 1.0 M potassium acetate (pH 5.2) eluent, with a flow rate of 1.0 ml/min and the column temperature set at 30 °C. A chromatogram obtained using these conditions is presented on Figure 2.10.

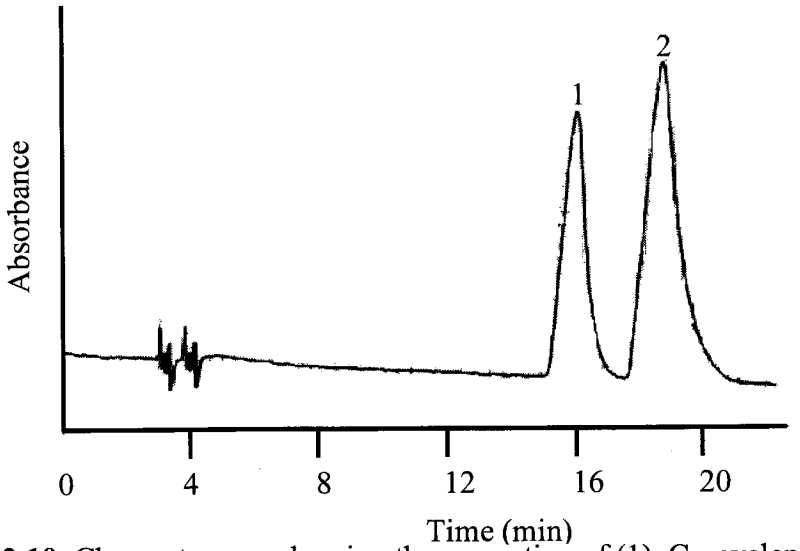


Figure 2.10. Chromatogram showing the separation of (1), Cu-cyclen; (2), Cu-cyclam by IEC.
Mobile phase: 10/90 (v/v) methanol/ 1.0 M potassium acetate at pH of 5.2
Flow rate: 1.0 mL/min Column: Partisil 10 SCX Detection at λ = 530 nm Temperature: 30°C

In order to measure the concentrations of Cu-cyclen or Cu-cyclam in a mixture a working curve is needed. The linear dynamic range for these complexes was established by running a set of calibration standards prior to analyzing the samples. The respective working curves for Cu-cyclen and Cu-cyclam are presented in Figures 2.11 and 2.12.

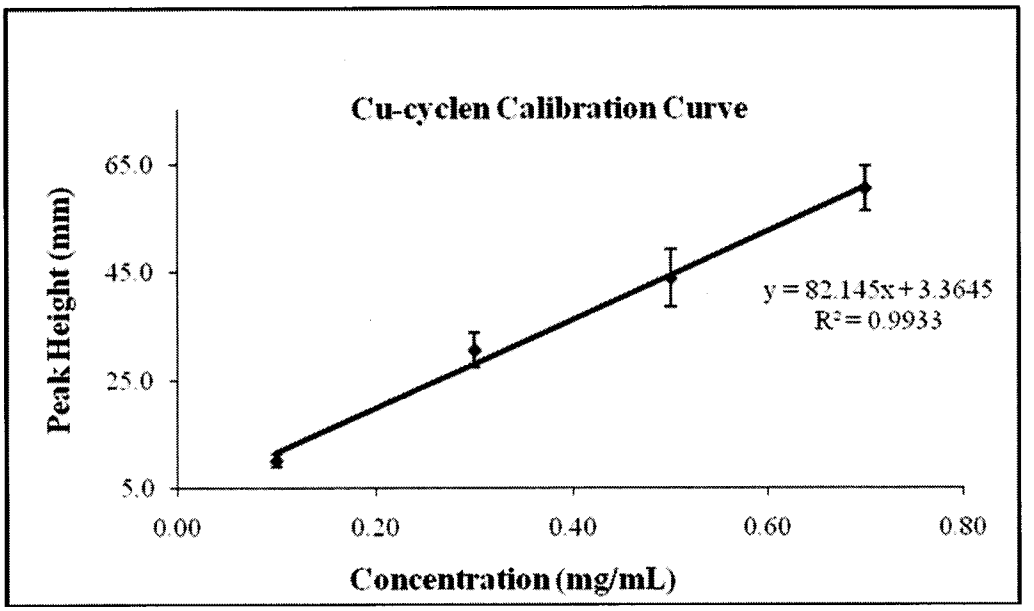


Figure 2.11. Working curve for Cu-cyclen produced using the same conditions as described in Figure 2.9.

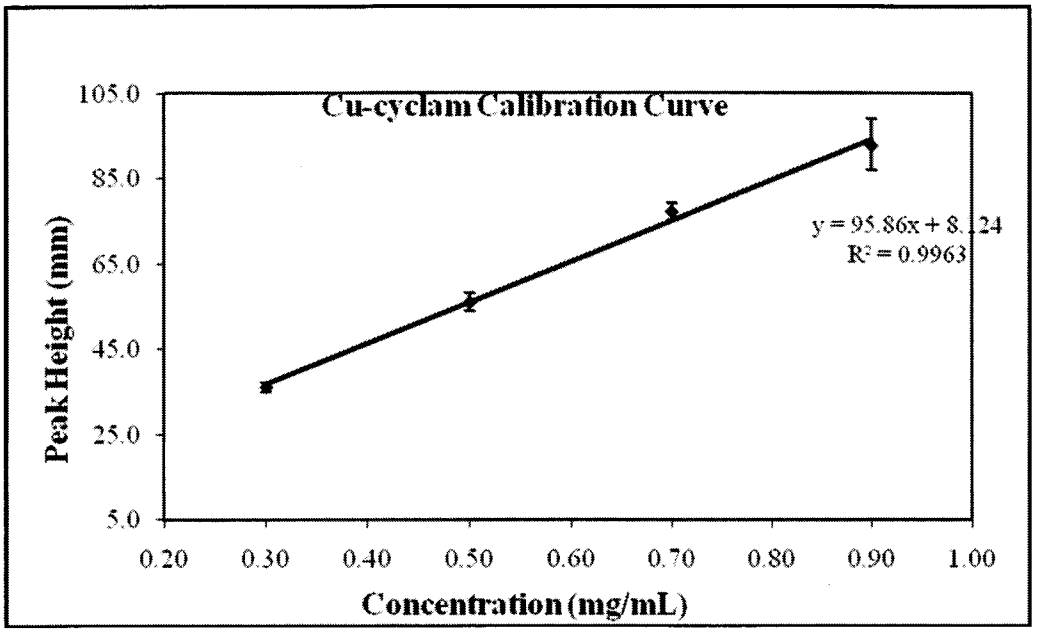


Figure 2.12. Working curve for Cu-cyclam produced using the same conditions as described in Figure 2.9.

For the equilibrium measurements a mixture of Cu-cyclen and cyclam were dissolved in aqueous solution. The initial concentrations of the starting materials in solution are given in Table 2.2.

Analyte	Initial concentrations	Equilibrium Concentrations
Cyclam	14.5 mM	6.88 mM
Cyclen	0	7.62 mM
Cu-cyclam-$(ClO_4)_2$	0	7.62 mM
Cu-cyclen-$(ClO_4)_2$	8.19 mM	0.57 mM

Table 2.2. Equilibrium data for a solution of Cu-cyclen and cyclam

A volume of 0.5 mL of the solution was taken and diluted with 2.0 mL of water to give a response in the linear dynamic range. Three injections of a diluted solution prior to heating showed there was no Cu-cyclam present in the sample. The aqueous solution of Cu-cyclen and cyclam was then heated at 60 °C for 24 hours. An aliquot of 0.5 mL was taken once the solution had cooled to room temperature. The 0.5 mL of the aqueous

mixture of Cu-cyclen and cyclam were then diluted with 2.0 mL of water. The solution was then heated at 90 °C for 24 hours and another aliquot was taken. The original sample was then heated for another 24 hours at 90 °C to ensure that equilibrium had been reached. The chromatograms showed no change in peak heights after the third day of heating, indicating that equilibrium had been reached.

The pH of the solution was measured and found to be 10.5 when the reaction was complete. The ionic strength of the solution was not controlled. The temperature at which equilibrium was achieved was 90 °C. The expected K'_f cannot be calculated accurately because the enthalpies and entropies of formation for the protonation of cyclen are not available. For this reason, the pK_as used in the calculations were not adjusted to account for temperature variation. The α values of the ligands were calculated using Equation 2.9 (given in Section 2.1) at a pH of 10.5. At 25 °C they were determined to be $\alpha_{cyclam} = 1.02 \times 10^{-1}$ and $\alpha_{cyclen} = 4.71 \times 10^{-1}$. The K'_fs need to be adjusted for temperature prior to adjusting them for pH. Using Equation 2.15 we can account for the effect of temperature on the formation constant. For Cu-cyclam the calculated $\Delta H°$ is 136 $kJmol^{-1}$ and $\Delta S°$ is 66.9 $Jmol^{-1}K^{-1}$ and for Cu-cyclen the calculated $\Delta H°$ is 95.0 $kJmol^{-1}$ and $\Delta S°$ is 155 $Jmol^{-1}K^{-}$.[31] The values of the calculated parameters and the conditional formation constants are given in Table 2.3.

$$\ln K = \frac{-\Delta H^{\circ}}{RT} + \frac{\Delta S^{\circ}}{R} \quad \textbf{(Equation 2.15)}$$

Parameter	Cyclam	Cyclen
α	0.0102	0.0471
$\Delta S°$	136 $KJmol^{-1}$	95 $KJmol^{-1}$
ΔH	66.9 $KJmol^{-1}K^{-1}$	155 $KJmol^{-1}K^{-1}$

Table 2.3. Parameters used to obtain the K'_f for Cu-cyclen and Cu-cyclam

The conditional formation constants of Cu-cyclam and Cu-cyclen adjusted for the effect of temperature are 9.92×10^{22} and 5.60×10^{21}, respectively. To obtain the conditional formation constant expected at 90 °C the effect of pH also needs to be taken into account. The calculations for the conditional formation constants for the Cu-cyclam and Cu-cyclen are given below. In addition K_3 the experimental equilibrium constant can be calculated using Equation 2.5.

$$K'_{\text{theoretical Cu-cyclam}} = \alpha_{\text{cyclam}} \times K_{\text{cyclam}}$$

$$= 1.02 \times 10^{-1} \times 9.92 \times 10^{22}$$

$$= 1.01 \times 10^{22}$$

$$K'_{\text{theoretical Cu-cyclen}} = \alpha_{\text{cyclen}} \times K_{\text{cyclen}}$$

$$= 4.71 \times 10^{-1} \times 5.60 \times 10^{21}$$

$$= 2.64 \times 10^{21}$$

$$K_3 = \frac{[7.62\text{mM}][7.62\text{mM}]}{[0.57\text{mM}][6.88\text{mM}]}$$

$K_3 = 14.8$

Using the experimental equilibrium constant we can calculate the predicted conditional formation constant for Cu-cyclen. Equation 2.6 is used for this calculation.

$$K_{ExpCu-cyclen} = \frac{1.01 \times 10^{22}}{14.8}$$

$K'_{\text{Experimental Cu-cyclen}} = 6.82 \times 10^{20}$

The K_3 calculated as shown in Equation 2.4 is 14.8. In Equation 2.5 substituting for the theoretical value of Cu-cyclam the conditional formation constant for Cu-cyclen based on experimental data is calculated. The experimental conditional formation constant for Cu-cyclen is 6.82×10^{20}. For ease of use in the literature log K'_f is used instead of the conditional formation constants. The theoretical value of logK'_f is 21.422 and the experimental logK'_f is 20.834. The difference in log conditional formation constants is 0.589 or a -2.74% error from the theoretical conditional formation constant. This calculation does not take into account the effect of the ionic strength in the solution. These results, however, demonstrate the viability of HPLC for the determination of conditional formation constants for copper tetraazamacrocyclic complexes.

2.3.2b Attempted competitive ligand binding

Determining the conditional complex formation constant of Cu-CB-TE2A within the physiological pH range is of interest so it can be compared to the known values of other copper complexes. These data will be useful in evaluating the potential of this complex for radiopharmaceutical applications. The conditional formation constant for Cu-CB-TE2A could not be determined at a pH of 7.4, 0.1 M ionic strength, and at 95 °C due to slow kinetics. A mixture of Cu-CB-TE2A and cyclam was prepared. The sample was heated for three weeks at 95 °C with only minor changes in the concentrations of Cu-CB-TE2A and Cu-cyclam, which indicates that Cu_CB-TE2A was being formed at slow rates. Equilibrium between Cu-cyclam and CB-TE2A was not achieved either. There

was a change in the concentration of Cu-cyclam which decreased as the concentration of Cu-CB-TE2A increased during the first week. However there were only small changes in the following days and the mixture failed to achieve equilibrium after six weeks of heating at 95 °C. The transchelation of copper (II) from the Cu-CB-TE2A to cyclam is therefore a slow process even at high temperatures. In order to force the complexes to achieve equilibrium in a reasonable time a microwave reactor was used to heat up the solution. The reaction mixture was heated at 150 °C using the microwave reactor for 6 hours. It was not possible to bring the reaction to an equilibrium as peak heights continued to change. The mixture was then heated at a high temperature (250 °C) which resulted in degradation. The solution had a black color and several impurities appeared on the chromatogram. The impurities were not identified as it was highly likely that the sample had degraded significantly. The presence of impurities would have interfered with the equilibrium calculations. One assumption being made is that copper will be bound to either CB-TE2A or cyclam but based on the chromatographic profiles that might not be the case for the Cu-CB-TE2A solution after it was heated at 250 °C.

In addition to Cu-CB-TE2A and cyclam, other competition reactions were investigated. A solution containing Cu-CB-TE1A and cyclam was heated for over six weeks without any significant changes. Cu-CB-cyclam and cyclam two closely related complexes were also investigated. There was no significant change in the concentration of the complexes after a four week period. The time needed for these mixture to reach equilibrium is extremely long, making it impractical to determine the conditional formation constants using this methodology. Another approach should be utilized to obtain conditional formation constants for copper complexes with cross-bridged ligands.

For example, a possibility is the addition of a transfer ligand like iminodiacetic acid which can facilitate the transfer of the copper from one complex to the other.

2.4 Conclusion

The chromatographic behavior of several complexes was evaluated on strong cation exchange and PGC stationary phases. For the +2 charged complex cations of Cu-cyclam, Cu-cyclen, Cu-CB-cyclam, and Cu-CB-cyclen, it was determined that the strong cation exchange stationary phase provides the best separation. For the +1 charged complex cations of Cu-CB-TE1A and Cu-CB-TEAMA, a separation can be obtained on both a strong cation exchange column and on a PGC stationary phase. The use of the PGC stationary phase allows for the charged complexes to be separated from neutral compounds. It was shown that chromatographic conditions can be used to determine conditional formation constants. However due to the extremely slow exchange kinetics it was not feasible to obtain conditional formation constant data for the selected cross-bridged complexes investigated.

CHAPTER III

EVALUATION OF CHROMATOGRAPHIC PROPERTIES OF SELECTED COPPER COMPLEXES WITH CROSS-BRIDGED TETRAAZAMACROCYCLIC LIGANDS AND DETERMINATION OF PHYSICOCHEMICAL PARAMETERS

3.1 Introduction

The Weisman-Wong research group recently synthesized four second generation chelators with methane-phosphonate pendant arms. The structures of the copper complexes, Cu-CB-DO2P^{OEt}, Cu-CB-TE2P^{OEt}, Cu-CB-TE2P, and Cu-CB-TE1A1P which have been synthesized using these chelators are shown in Figure 3.1.

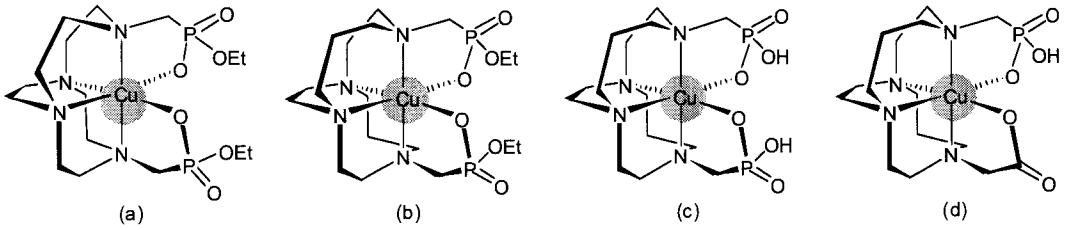


Figure 3.1. Structures of (a), Cu-CB-DO2P^{OEt}; (b), Cu-CB-TE2P^{OEt}; (c), Cu-CB-TE2P; (d), Cu-CB-TE1A1P

The chromatographic behaviors of several related Cu (II) complexes of

di-carboxymethyl-armed ligands, including CB-DO2A and CB-TE2A, have been studied previously by Shen. [35] In the work reported here, the effect of macrocyclic ring size, 12- versus 14-membered, on the chromatographic behavior of Cu-CB-TE2P^{OEt} and Cu-CB-DO2P^{OEt} was evaluated. Cu- CB-TE2P^{OEt} complex has two additional ethyl groups on the pendant arms in comparison to Cu-CB-TE2P, thus it allows the evaluation of chromatographic properties based on hydrophobicity. The nature of pendant arm methane-phosphonate versus carboxymethyl is also expected to affect the retention behavior of the complexes on different stationary phases including C_{18}, porous graphitic carbon (PGC) and silica hydride. The chromatographic behavior of Cu-CB-TE2P, Cu-CB-TE1A1P and Cu-CB-TE2A was evaluated in order to identify any trends present due to the type of pendant arm. Cu-CB-TE2P has two methane phosphonate arms, Cu-CB-TE1A1P has one methane phosphonate and one acetate arm, and Cu-CB-TE2A has two acetate arms. The pH of the mobile phase is also expected to affect the retention behavior of Cu-CB-TE2P and Cu-CB-TE1A1P due to the ionizability of the methane phosphonate arms. The Cu-CB-TE2P is expected to be pH active in the range from 3 to 8 while the Cu-CB-TE1A1P is expected to be pH active in the range from 5 to 9. The development of separation conditions for these complexes allows for evaluation of their purity, measurement of physico-chemical parameters and prediction of their *in vivo* behavior.

The acid dissociation constant (K_a) is a key parameter affecting important properties of the copper complex including solubility, lipophilicity and permeability through a membrane.[26] It is important to remember that a small change in pH can cause a large change in the percentage of the complex which is ionized if the pH of the solution

and the pK_a of the complex are close together. Therefore, it is important to study the acid dissociation constants as they reveal the proportions of the different ionic species present in the analyte solution at any given pH. For example different ionic species have different UV-Vis molar absorptivities. Therefore, when conducting UV-Vis experiments it is important to choose a pH where only one ionic species is present in solution. Kinetic inertness experiments for the complexes at high temperatures or acidic pH, use UV-Vis detection. In addition this information can be used when synthesizing the complexes. The synthesis of the complex should be performed at a pH where the complex will be neutral as it will be easier to isolate the desired product.

In reversed phase chromatography the behavior of the analyte changes depending on the charge of the analyte. Typically on a C_{18} stationary phase a neutral analyte is retained longer than either a positively or negatively charged analyte. In order to change the ionization state of the analyte of interest the pH of the mobile phase is varied. One of the complexes being studied, Cu-CB-TE2P, is a diprotic acid. A goal of this study was to determine the two K_a values for Cu-CB-TE2P by observing changes in its chromatographic behavior.

The octanol/water partition coefficient, $K_{o/w}$, is considered to be the standard in terms of lipophilicity parameter estimation. The $K_{o/w}$ is determined using the so called "shake flask" method.[71] The concentration of the solute in both phases is determined after the addition of the analyte to the partitioning solvents (1-octanol, water) in a separatory funnel with shaking to accelerate the partitioning equilibrium. The partition coefficient between an organic and aqueous phase is known as "P" and is calculated

according to the formula shown in Equation 3.1. In the previously mentioned example "P", is equal to the ratio of the concentration of the analyte in the organic phase, 1-octanol, to that of the concentration of analyte in the aqueous phase as defined in Equation 3.2. For ease of use the values of lipophilicity reported in literature are log P.

$$P = C_{organic}/C_{aqueous} \qquad \textbf{(Equation 3.1)}$$

$$P = K_{o/w} = C_{1\text{-octanol}}/C_{water} \qquad \textbf{(Equation 3.2)}$$

The "shake flask" method is conceptually very simple but in practice there are many concerns and limitations. The most important limitations are the ability to: control temperature, determine the length of the equilibration process, and estimate the volumes of the two phases for easy detection and analyte concentration determination. Additional limitations include: limited interlaboratory reproducibility, difficulty in determining log P for ionic compounds, and the formation of emulsions. [71,72]

The observation of a linear relationship between log P and log k'_w, (k'_w is the capacity factor of analyte eluted with 100% aqueous mobile phase) has made it possible for the later to become a reasonable substitute. [72] For example Kaliszan demonstrated a good correlation between log P and log k'_w for a series of compounds which included carbazole, cumene, coumarin, and anthracene.[73] Recently, the pharmaceutical industry has adopted the use of chromatographic methods for determination of the lipophilicity parameters.[75] As mentioned in Chapter I of this thesis, HPLC offers several advantages over the "shake flask" method including reduced analysis time and sample handling, smaller sample size, greater sensitivity, and reproducibility. [47]

An additional goal of this study was to compare the lipophilicity parameters for

Cu-CB-DO2P^{OEt}, Cu-CB-TE2P^{OEt}, Cu-CB-TE2P, and Cu-CB-TE1A1P (Figure 3.1) to those obtained for Cu-CB-TE2A and Cu-CB-DO2A (Figure 1.10). In this study a C_{18}, a porous graphitic carbon (PGC), and a silica hydride stationary phase were evaluated for determining the lipophilicity parameters of Cu-CB-DO2P^{OEt}, Cu-CB-TE2P^{OEt}, and Cu-CB-TE2P. The retention of each complex is measured using a completely aqueous mobile phase to determine the lipophilicity parameter. The reason for using a totally aqueous mobile phase is so that the partitioning of the analyte between the stationary and mobile phase to be close to that of octanol/water. No organic modifier, other than the required buffer, was present in the mobile phase used to elute the copper complexes on the C_{18} stationary phase. In this study the six complexes, Cu-CB-DO2A, Cu-CB-TE2A, Cu-CB-DO2P^{OEt}, Cu-CB-TE2P^{OEt}, Cu-CB-TE2P, and Cu-CB-TE1A1P were eluted with a 10 mM citrate (pH at 2.5) mobile phase from a C_{18} stationary phase to determine their k'_w.

The effect of stationary phase type on the measured lipophilicity parameters was evaluated by studying the retention properties of the copper complexes on C_{18}, PGC, and silica hydride stationary phases. Hydrophobic interactions are expected to be the most important factor influencing the retention of analytes in reversed phase chromatography. Therefore, the overall trend of the analytes' capacity factors should be similar among different stationary phases utilized in a reversed phase mode.

When using a C_{18} stationary phase, retention discrimination of the analytes is based mainly on hydrophobicity. However on the PGC stationary phase there are also electronic and polar type interactions, besides hydrophobic ones, which affect the

chromatographic behavior of the analytes. The properties and applications of the PGC stationary phase were discussed in more detail in Chapter II.

The other stationary phase being used is based on a silica hydride support minimally modified with carbon. Silica hydride stationary phases were not extensively 3used until the 1990s.[71-80] However, following recent improvements they have found a wide range of application including the separation of polar compounds such as amines (creatine, creatinine, alanine, aspargine), carbohydrates (fructose, sorbitol, glucose), organic acids and nucleotides. [75-78] The diamond hydride stationary phase which is being used in this study has been shown to provide separation of amino acids, and organic acids such as fumaric and maleic acid.[79] In addition this stationary phase has been used for the separation of serotonin including its metabolites and analogs.[80] There are some differences between the silica hydride and silica based stationary phases. For example, at least 95% of the surface is populated by the nonpolar silicon-hydride (Si-H) groups on the silica hydride stationary phase. [81] The enhanced stability of the Si-H bond arises from the stabilization of the larger polymer matrix of the silica. Chromatographic and spectroscopic studies have confirmed the stability of this bond.[81] The silicon hydride groups result in a stationary phase which has unique properties including enhanced retention of non-polar analytes. Additionally the presence of the silicon hydride bonds results in a stationary phase with enhanced stability at low pH (~ 2) and also at high pH (~ 9-10). [75-78]

The silica hydride phase has been modified and alkynes and cyano groups have been bonded to the support. In addition the silica hydride support can be loaded with carbon as is the case with the diamond hydride stationary phase used for this study. The

properties of the silica hydride stationary phase depend on the type of moiety attached to the support. For example when an alkyl moiety, C_{18}, is bound to the silica hydride the stationary phase has capabilities which are similar to commercial C_{18} silica bonded stationary phases. [75-78] The differences between the support materials (Si-H vs. silica) give rise to unique selectivity when utilizing the silica hydride based stationary phase. [75]

There have been reports that silica hydride based stationary phase can act as both a normal and a reversed phase stationary phases. [80] Pesek was able to separate a group of closely related phenols using an organic mobile phase consisting of 10/90 (v/v) diethyl ether/hexanes.[80] The same stationary phase was used to separate a group of closely related carbohydrates, eluting them with 100% aqueous mobile phase. One of the main characteristics of this stationary phase is that different solvents may drastically affect the retention behavior of the analytes. Pesek noted that an aprotic solvent such as acetonitrile affected retention of amines much more than a protic solvent such as methanol.[75]

The chromatographic behavior of selected copper complexes: Cu-CB-TE2A, Cu-CB-TE2P, Cu-CB-TE1A1P, Cu-CB-TE2P^{OEt}, and Cu-CB-DO2P^{OEt} (Figures 1.10 and 3.1) was investigated for a diamond hydride stationary phase. The properties of this stationary phase are expected to afford enhanced retention for the copper complexes of interest. It has been shown that this stationary phase exhibits increased retention toward compounds containing phosphonate groups.[75] Therefore the second generation of chelators featuring the methanephosphonate arms should have a larger capacity factor compared to those obtained on the C_{18}.

3.2 Experimental

3.2.1 Reagents

ACS certified anhydrous citric acid, sodium borate, sodium monobasic phosphate monohydrate, ACS grade sodium hydroxide, reagent grade potassium nitrate and potassium phthalate were purchased from Fisher (Fairlawn, NJ, USA). HPLC-grade methanol was purchased from Pharmco (Brookfield, NJ, USA). The DI water used for the preparation of the standards and the eluents was obtained from a Milli-Q Water System. All mobile phases were vacuum filtered through a 0.45 μm nylon filter (Whatman, OR, USA) prior to use.

3.2.2 Apparatus

Chromatographic separations were performed using a Varian 9010 pump fitted with a Rheodyne 7125 injector (Cotati, CA, USA) having a 10 μL injection loop. UV detection was performed using a Waters 486E UV-Vis absorbance detector (Milford, MA, USA) with the detection wavelength set at 280 nm. The detector was interfaced to a laboratory computer data system or a Kipp and Zonen BD41 chart recorder. The columns were held at a temperature of 25° C using a column heater (Jones Chromatography, Hengoed, UK). The retention properties of the copper complexes were investigated using four HPLC stationary phases: (1) Betabasic C_{18} (150 × 4.6 mm; 5μm; Thermo Scientific, Waltham, MA, USA); (2) Agilent SB-C_{18} (50 × 4.6 mm; 3.5μm; Agilent, Wilmington, DE, USA); (3) Porous Graphitic Carbon [Hypercarb] (100 × 3.0 mm; 5.0μm; Thermo Scientific, Waltham, MA, USA); and (4) Cogent Diamond Hydride (100 × 4.6 mm; 4μm;

Microsolv Technologies, Eatontown, NJ, USA). A Vernier pH probe was used during the preparation of the citrate buffer and for measuring the pH of the mobile phase.

3.2.3 Chromatographic Conditions

A sodium hydroxide solution was prepared by diluting about 40 g of NaOH in CO_2 free DI water. The sodium hydroxide solution was standardized using potassium hydrogen phthalate. The standardized sodium hydroxide solution was added using a burette, to adjust the pH of the buffers. The mobile phase for each experiment was prepared by diluting the necessary volume of buffer to produce a 10mM solution of that buffer. Sodium nitrate was added to the pK_a runs to adjust the ionic strength to 0.1. The reason for adjusting the ionic strength is to allow the comparison of chromatographic data with the data obtained using potentiometric techniques. In potentiometric techniques the ionic strength is in most cases adjusted to 0.1. Each chromatographic column was equilibrated for at least 30 column volumes with the mobile phase to be used for the experiment. The columns were flushed with at least 30 column volumes of 70/30 methanol/water (v/v) solution after daily use. The dead time (t_0) used for the calculation of the capacity factor (k') was taken as the elution time of acetone when using a 100% methanol as the mobile phase for each of the four columns.

3.2.4 Sample Preparation

Cu(II)-CB-DO2A, Cu(II)-CB-TE2A, Cu(II)-CB-DO2P^{OEt}, Cu(II)-CB-TE2P^{OEt},

Cu(II)-CB-TE2P, and Cu(II)-CB-TE1A1P were synthesized by the Weisman-Wong group according to published methods.[38] Solutions of the complexes were prepared in deionized water for analysis by HPLC.

3.3 Results and Discussion

3.3.1 Evaluation of the chromatographic behavior of Cu-CB-DO2P^{OEt}, Cu-CB-TE2P^{OEt}, Cu-CB-TE1A1P, and Cu-CB-TE2P

In this section the parameters influencing the chromatographic behavior of selected copper complexes will be discussed. The differences in their structural properties including pendant arm functionalities and ring size affect their chromatographic behavior. Specifically the effect that the methane-phosphonate arms have on the retention behavior was investigated by comparing the capacity factors of Cu-CB-TE2A, Cu-CB-TE2P and Cu-CB-TE1A1P. Cu-CB-TE2A features carboxymethyl pendant arms whereas Cu-CB-TE2P and Cu-CB-TE1A1P feature phosphonate ones resulting in different interactions with the stationary phase. The pH of the mobile phase affects retention of Cu-CB-TE2P and Cu-CB-TE1A1P since they are both ionizable in aqueous solution. Elution with a 10mM citrate buffer (at pH 2.5) from the Betabasic C_{18} (150 × 4.6 mm; 5μm) stationary phase revealed that the neutral form of Cu-CB-TE2P has the lowest retention (t_R=5.7 minutes), Cu-CB-TE2A is in the middle (t_R=10.2 minutes) and Cu-CB-TE1A1P has the highest retention(t_R=15.4 minutes). The capacity factors values calculated using Equation 1.1 for the undissociated forms of the Cu-CB-TE2P, Cu-CB-TE2A and Cu-CB-TE1A1P complexes are 5.2, 5.9 and 8.5 respectively. Representative

chromatograms for Cu-CB-TE2A, Cu-CB-TE2P and Cu-CB-TE1A1P under the same elution conditions are shown in Figure 3.2.

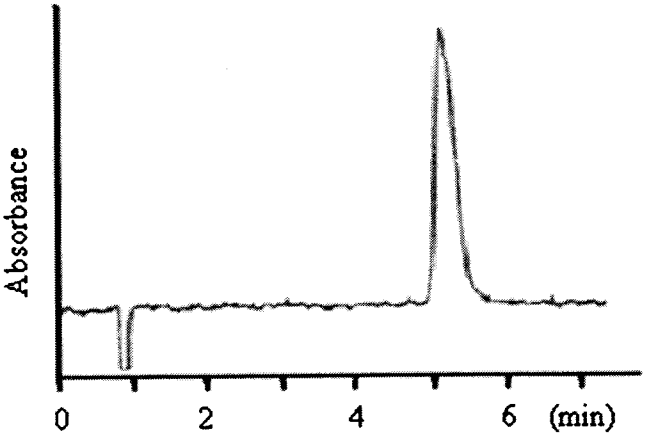


Figure 3.2.a. Chromatogram of Cu-CB-TE2P injected at a concentration 0.10 mg/mL
Mobile Phase: 10 mM citrate at pH 2.5 added $NaNO_3$ to adjust ionic strength
Flow rate: 2.0 mL/min Column: Agilent C_{18} (50 × 4.6 mm; 3.5μm)
Detection λ: 280 nm Temperature: 25 °C Injection: 10 μL

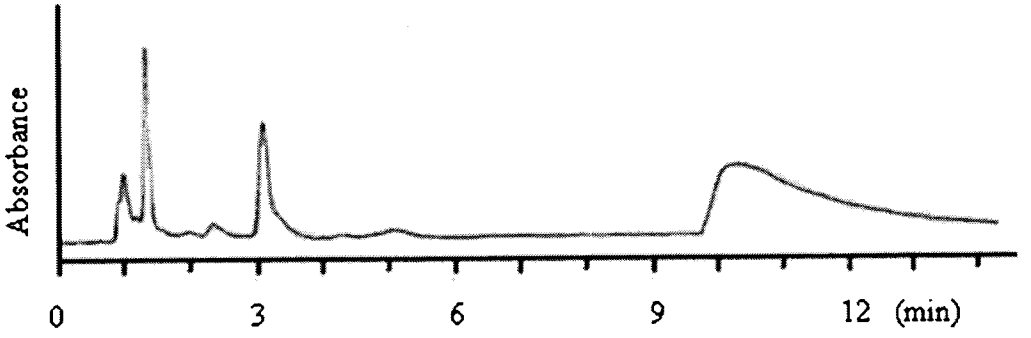


Figure 3.2.b. Chromatogram of Cu-CB-TE2A injected at a concentration 0.10 mg/mL.
Mobile Phase: 10 mM citrate at pH 2.5 added $NaNO_3$ to adjust ionic strength
Flow rate: 1.2 mL/min Column: Betabasic C_{18} (150 × 4.6 mm; 5μm)
Detection λ: 280 nm Temperature: 25 °C Injection: 10 μL

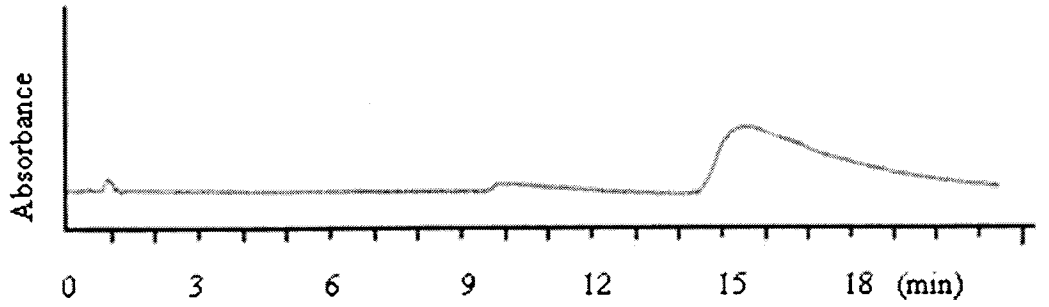


Figure 3.2.c. Chromatogram of Cu-CB-TE1A1P injected at a concentration 0.10 mg/mL
Mobile Phase: 10 mM citrate at pH 2.5 added $NaNO_3$ to adjust ionic strength
Flow rate: 1.2 mL/min Column: Betabasic C_{18} (150 × 4.6 mm; 5μm)
Detection λ: 280 nm Temperature: 25 °C Injection: 10 μL

The peak shapes for these three complexes show tailing as can be seen in Figure 3.2.a-c. Cu-CB-TE1A1P (Figure 3.2.c) has the worst peak shape. The peak shapes are improved by the addition of an organic modifier to the mobile phase. The fully dissociated form (-2 charged) of Cu-CB-TE2P has a capacity factor ($k'_{Cu\text{-}CB\text{-}TE2P2\text{-}}=1.5$) which as expected is smaller than that of Cu-CB-TE2A which is neutral ($k'_{Cu\text{-}CB\text{-}TE2A}=5.9$) on the Betabasic C_{18} stationary phase using 10mM citric acid as an eluent (as shown in Figure 3.3b and 3.2b). The -1 charged species of Cu-CB-TE1A1P demonstrates similar behavior as that of the charged Cu-CB-TE2P (Figure 3.3a and c). Both dissociated forms of the complexes are less retained than the undissociated form.

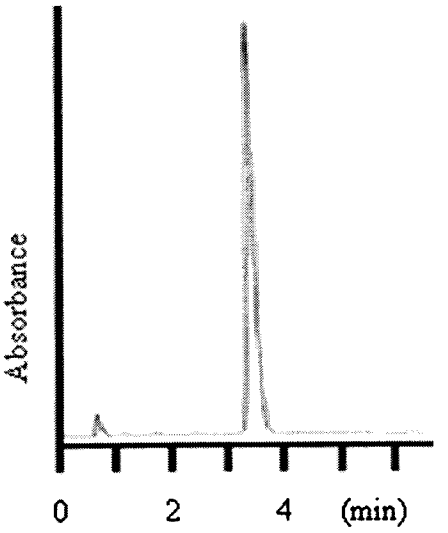


Figure 3.3.a. Chromatogram of Cu-CB-TE2P^{1-} injected at a concentration 0.10 mg/mL
Mobile Phase: 10 mM citrate at pH 6.0 added $NaNO_3$ to adjust ionic strength
Flow rate: 0.7 mL/min Column: Agilent C_{18} (150 × 4.6 mm; 5μm)
Detection λ: 280 nm Temperature: 25 °C Injection: 10 μL

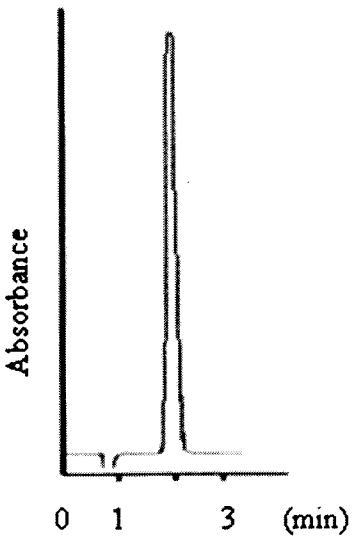


Figure 3.3.b. Chromatogram of Cu-CB-TE2P^{2-} injected
at a concentration 0.10 mg/mL
Mobile Phase: 10 mM borate at pH 9.82 added $NaNO_3$ to adjust ionic strength
Flow rate: 0.7 mL/min Column: Agilent C_{18} (150 × 4.6 mm; 5μm)
Detection λ: 280 nm Temperature: 25 °C Injection: 10 μL

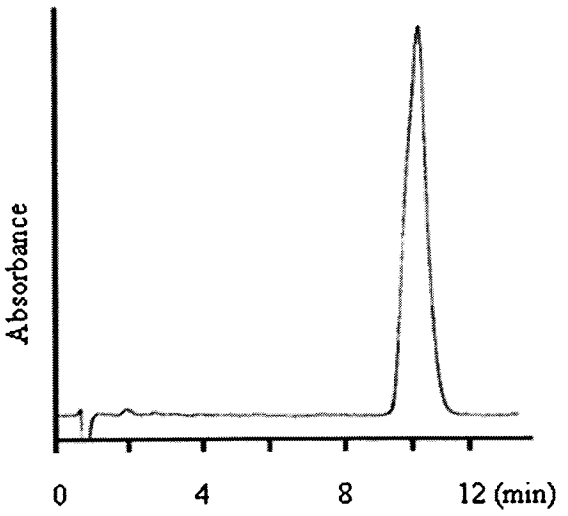


Figure 3.3.c Chromatogram of Cu-CB-TE1A1P^{1-} injected at a concentration 0.10 mg/mL
Mobile Phase: 10 mM citrate at pH 9.82 added $NaNO_3$ to adjust ionic strength
Flow rate: 0.7 mL/min Column: Agilent C_{18} (150 × 4.6 mm; 5μm)
Detection λ: 280 nm Temperature: 25 °C Injection: 10 μL

The difference between Cu-CB-DO2P^{OEt} and Cu-CB-TE2P^{OEt} is the ring size, the latter complex has two additional methylenes in the macrocyclic ring. Cu-CB-TE2P^{OEt} is the more hydrophobic complex and is more strongly retained on a typical C_{18} stationary phase. Shen observed this behavior previously while studying the behavior for Cu-CB-TE2A which eluted later than Cu-CB-DO2A since Cu-CB-TE2A also has two additional methylenes in the macrocyclic ring.[33] The experiments confirmed that Cu-CB-TE2P^{OEt} with has larger ring size will elute later. Cu-CB-TE2P^{OEt} ($k'_{Cu\text{-}CB\text{-}TE2POEt}$ = 65.0) has a capacity factor that is about 4 times larger than that of Cu-CB-DO2P^{OEt} ($k'_{Cu\text{-}CB\text{-}TE2POEt}$ = 16.0) when using a 3/97 (v/v) methanol/10mM citrate (pH 2.5) as the eluent on a Betabasic C_{18} stationary phase (Figures 3.4.a and b).

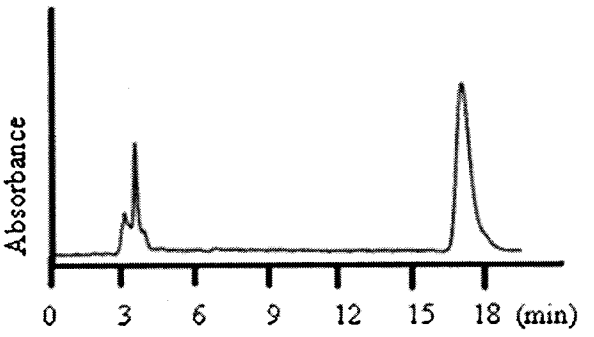


Figure 3.4.a. Chromatogram of Cu-CB-DO2P^{OEt}
Mobile Phase: 3/97 (v/v) methanol/10 mM citrate (pH 2.5) Flow rate: 1.7 mL/min
Column: Betabasic C_{18} (150 × 4.6 mm; 5μm) Detection λ: 280 nm
Temperature: 25 °C Injection: 10 μL of 0.5 mg/mL of Cu-CB-DO2P^{OEt}

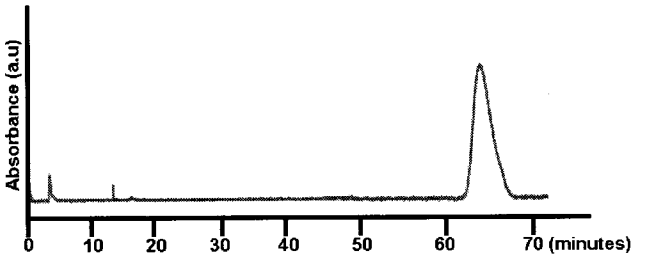


Figure 3.4.b. Chromatogram of Cu-CB-TE2P^{OEt}
Mobile Phase: 3/97 (v/v) methanol/10 mM citrate (pH 2.5) Flow rate: 1.7 mL/min
Column: Betabasic C_{18} (150 × 4.6 mm; 5μm) Detection λ: 280 nm
Temperature: 25 °C Injection: 10 μL of 0.5 mg/mL of Cu-CB-TE2P^{OEt}

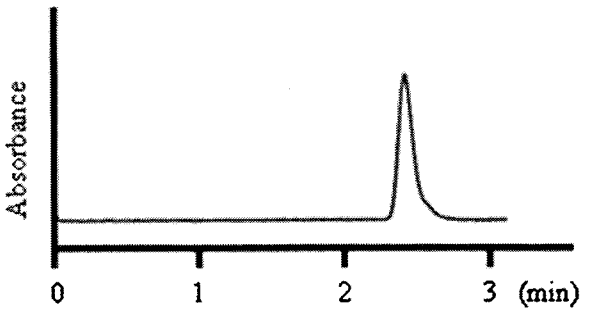


Figure 3.4.c. Chromatogram of Cu-CB-TE2P
Mobile Phase: 3/97 (v/v) methanol/10 mM citrate (pH 2.5) Flow rate: 1.7 mL/min
Column: Betabasic C_{18} (150 × 4.6 mm; 5μm) Detection λ: 280 nm
Temperature: 25 °C Injection: 10 μL of 0.5 mg/mL of Cu-CB-TE2P

The peak shapes improve with the addition of methanol to the mobile phase. Methanol wets the alkyl chain present in the stationary phase and reduces band broadening, even when added in small amounts as shown, in Figures 3.4.a-c. In addition to improving peak shape methanol, as expected, reduced the analysis time for the complexes.

Experiments revealed that Cu-CB-TE2P^{OEt} having pendant arms which have additional ethyl ester units was retained more on the Betabasic C_{18} in comparison to Cu-CB-TE2P. For example the capacity factor of Cu-CB-TE2P^{OEt} ($k'_{Cu\text{-}CB\text{-}TE2POEt}$ = 65.0) is 45 times larger than that of Cu-CB-TE2P ($k'_{Cu\text{-}CB\text{-}TE2P}$ =1.46) when using a 3/97 (v/v) methanol/10mM citrate (pH 2.5) as the eluent on a Betabasic C_{18} stationary phase (Figures 3.4.a and c).

A comparison of the chromatographic behavior of Cu-CB-DO2P^{OEt} and Cu-CB-TE2P allows for a better understanding of the retention mechanism for these complexes. The data show that the modified arms of Cu-CB-DO2P^{OEt} which have an ethyl ester moiety are responsible for additional interactions with the Betabasic C_{18}

stationary phase. The capacity factor for Cu-CB-DO2P^{OEt} ($k'_{Cu\text{-}CB\text{-}DO2POEt}$ =16.0) is about 11 times greater than that of Cu-CB-TE2P ($k'_{Cu\text{-}CB\text{-}TE2P}$ =1.46) when using a 3/97 (v/v) methanol/10mM citrate (pH 2.5) as the eluent on a Betabasic C_{18} stationary phase (Figures 3.4.b and c). These results indicate that pendant arm type affects the capacity factor to a greater degree in comparison with ring size under reversed phase conditions.

The retention of copper complexes with the methanephosphonate pendant arm ligands was investigated on a PGC stationary phase as well. Previous work by Shen revealed enhanced retention for Cu-CB-DO2A and Cu-CB-TE2A complexes on a PGC stationary phase under reversed phase conditions compared to typical C_{18} stationary phase.[34] The capacity factors for Cu-CB-TE2A, when using the PGC stationary phase with a mobile phase of 15/85(v/v) methanol/ 30 mM citrate buffer (pH 2.5) was comparable to that determined when using an 100% aqueous mobile phase containing a 10 mM citrate buffer (pH 2.5) on a C_{18} stationary phase. The same trend was confirmed for the analytes of interest in this study. For example Cu-CB-TE2P has a capacity factor at least 2 times higher on the PGC stationary phase compared to a C_{18} stationary phase under similar elution conditions. As early as the 1980's it was reported that PGC as a stationary phase offers enhanced electronic and polar-type interactions. [80] Increased retention as a result of π-π interactions between the analytes and this stationary phase has been reported.[81] In addition the PGC stationary phase has been reported to act as a Lewis base towards polar compounds. [63]

In this study it was also confirmed that it is possible for ionic compounds to be retained longer than neutral ones when using PGC as a stationary phase. For example, Cu-CB-TEAMA and Cu-CB-TE1A (Figure 1.2) are both +1 charged species in aqueous

solution below pH 8. Both these complexes exhibited increased retention on the PGC stationary phase compared to Cu-CB-TE2A as discussed in detail in Chapter II (see Figures 2.7 and 2.8).

The data show that the amphiprotic form of Cu-CB-TE2P is retained longer than the undissociated form of Cu-CB-TE2P as can be seen in Figures 3.5.a and b. The fact that charged complexes demonstrate enhanced retention indicates electronic type interactions are occurring between the complexes and the PGC stationary phase.

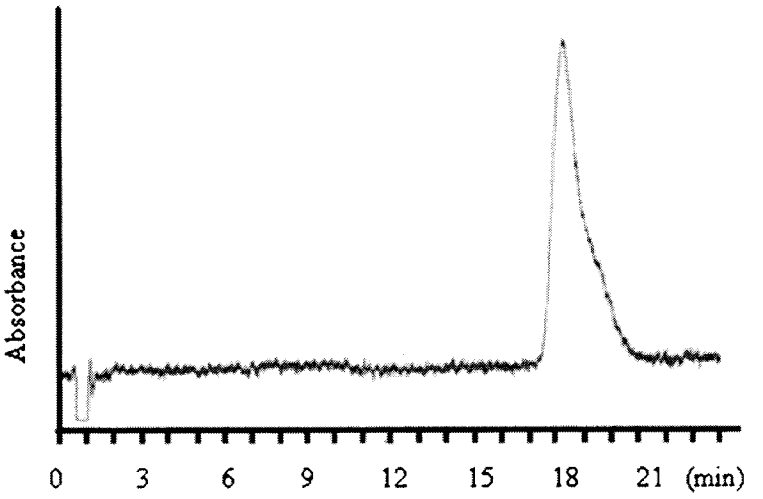


Figure 3.5.a. Chromatogram of the neutral form of Cu-CB-TE2P on the PGC column
Mobile Phase: 10 mM citrate (pH 2.73) Flow rate: 0.65 mL/min
Column: Porous Graphitic Carbon (100 × 3.0 mm; 5μm) Detection λ: 280 nm
Temperature: 25 °C Injection: 10 μL of 0.10 mg/mL of Cu-CB-TE2P

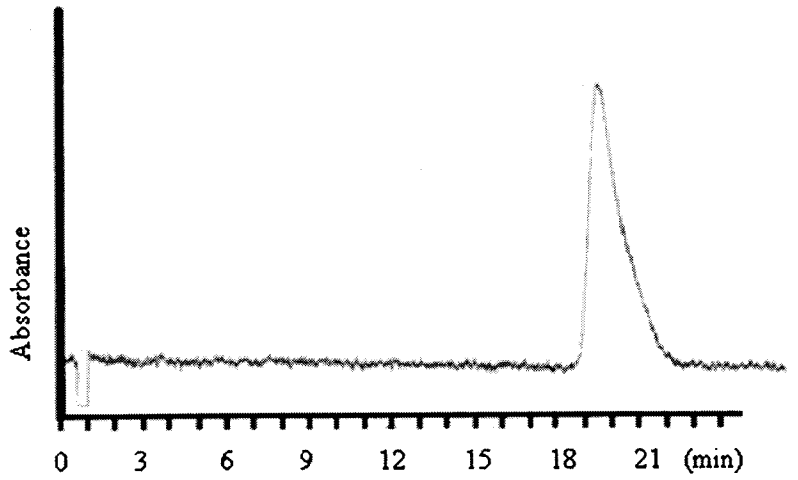


Figure 3.5.b. Chromatogram of the amphiprotic form Cu-CB-TE2P on the PGC column
Mobile Phase: 10 mM citrate (pH 3.46) Flow rate: 0.65 mL/min
Column: Porous Graphitic Carbon (100 × 3.0 mm; 5μm) Detection λ: 280 nm
Temperature: 25 °C Injection: 10 μL of 0.10 mg/mL of Cu-CB-TE2P

On the PGC stationary phase adjusting the mobile phase pH from 2.5 to 5.5 did not affect the retention behavior of Cu-CB-TE2P^{OEt} or Cu-CB-DO2P^{OEt} which demonstrates that the properties of the stationary phase do not change as a result of pH over the 2.5 to 5.5 range. On the other hand pH has an effect on the charge of Cu-CB-TE2P. The undissociated form of the Cu-CB-TE2P is the major species in solution at a mobile phase pH of 2.73. When the mobile phase pH is 3.46 there is at least 20 percent of the amphiprotic species present. The increased retention at this pH could be due to the presence of the charged species of Cu-CB-TE2P.

In addition to C_{18} and PGC stationary phases, the retention behavior for the methanephosphonate pendant arm copper complexes was investigated on a silica hydride stationary phase. This stationary phase provided the greatest retention for Cu-CB-DO2P^{OEt}, Cu-CB-TE2P^{OEt}, Cu-CB-TE2P, and Cu-CB-TE1A1P complexes when compared to the C_{18} and PGC stationary phases. The capacity factor for Cu-CB-TE2P

was at least three times larger on the silica hydride stationary phase in comparison to the PGC stationary phase under 10 mM citrate buffer (pH 2.5) conditions with no organic modifier. The other two complexes, Cu-CB-DO2P^{OEt} and Cu-CB-TE2P^{OEt}, were eluted with the addition of methanol to the mobile phase due to the increased retention. The capacity factors of these complexes on the modified silica hydride stationary phase were 2 to 3 times higher than those measured on the PGC stationary phase under the same mobile phase conditions. As was observed with the PGC stationary phase, the charged form of Cu-CB-TE2P eluted later than the neutral form of the complex. Chromatograms obtained on the silica hydride stationary for Cu-CB-TE2P are shown in Figure 3.6.a and b.

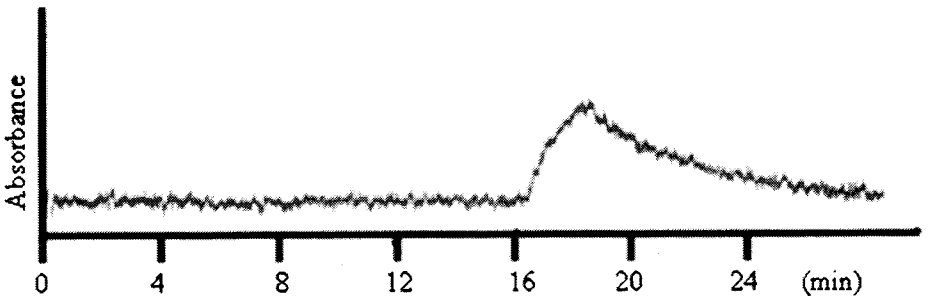


Figure 3.6.a. Chromatogram for the neutral form of Cu-CB-TE2P
Mobile Phase: 10 mM citrate at pH 2.73, added $NaNO_3$ to adjust ionic strength
Flow rate: 2.5 mL/min Column: Diamond Hydride (100 × 4.6 mm; 4μm)
Detection λ: 280 nm Temperature: 25 °C Injection: 10 μL

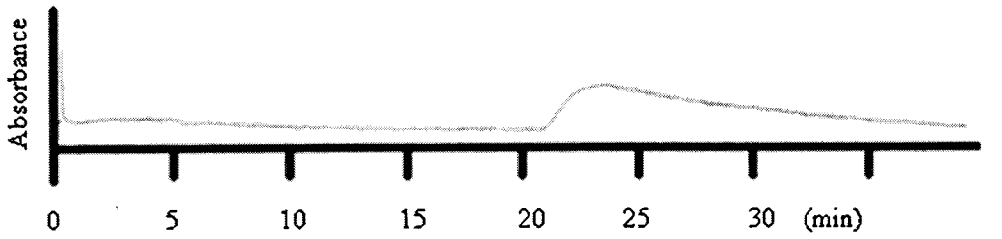


Figure 3.6.b. Chromatogram for the amphiprotic form of Cu-CB-TE2P
Mobile Phase: 10 mM citrate at pH 5.53, added $NaNO_3$ to adjust ionic strength
Flow rate: 2.5 mL/min Column: Diamond Hydride (100 × 4.6 mm; 4μm)
Detection λ: 280 nm Temperature: 25 °C Injection: 10 μL

The experimental results show that the capacity factor for Cu-CB-TE2P on the diamond hydride stationary phase is 5 times larger than that obtained on a C_{18} stationary phase under similar elution conditions. The primary interactions responsible for the retention of an analyte on a C_{18} are the hydrophobic ones. Therefore the reason for the increased retention on the diamond hydride stationary phase is possibly due to additional interactions of the analyte with this phase in addition to the hydrophobic interactions. One possibility is for some type of ion exchange retention mechanism since the charged complexes exhibited stronger interactions with the diamond hydride stationary phase. The other possibility is to have some type of electronic or polar retention mechanism similar to those observed on the PGC stationary phase.

3.3.2 Estimation of pK_as of Cu-CB-TE2P using C_{18} stationary phases

As mentioned in the Introduction section, the K_a values for Cu-CB-TE2P were estimated using chromatographic methods. The mobile phase pH ranged from 2.2 to 9.8

since Cu-CB-TE2P is expected to have two pK_as. The pH of the mobile phase determines the ionization state of the complex which in turn affects its retention on the stationary phase. The behavior of the stationary phase is assumed to be unaffected by the pH of the mobile phase. The behavior of Cu-CB-TE2P^{OEt} was investigated on both the Agilent and Betabasic C_{18} stationary phases. The data shown in Table 3.1 demonstrate that the capacity factor for this complex did not change as a function of pH. Therefore, it is reasonable to assume that any changes observed in the capacity factor of the Cu-CB-TE2P will be due to the different ionization states of the complex.

Nominal pH of the mobile phase	Capacity factor on Agilent C_{18} stationary phase	Capacity factor on Betabasic C_{18} stationary phase
2	153	157
6	154	158
9	154	158

Table 3.1. Effect of pH on the capacity factor of Cu-CB-TE2P^{OEt} using the Betabasic C18 stationary phase.

The shape of the capacity factor vs. pH profile resembles that of a pH potentiometric titration. As expected, the undissociated form of the complex elutes later than either one of dissociated species, which are anions. The results for the experiments using two different C_{18} stationary phases are shown in the Figures 3.7 and 3.8. The data for the plots shown in Figures 3.7 and 3.8 are given in Tables 3.2 and 3.3.

pH	k'Theory	k' experimental
9.82	1.2	1.2
8.74	1.2	1.2
8.07	1.2	1.5
7.83	1.2	1.8
6.78	1.6	2.1
6.00	2.2	2.6
5.4	2.7	2.8
4.84	3.1	3.0
4.18	4.0	3.8
3.83	4.6	4.6
3.44	5.2	5.4
3.19	5.4	5.5
2.93	5.6	5.6
2.74	5.7	5.7
2.63	5.7	5.8

Table 3.2. The capacity factors for the Cu-CB-TE2P complex as a function of mobile phase pH on a Agilent C_{18} stationary phase

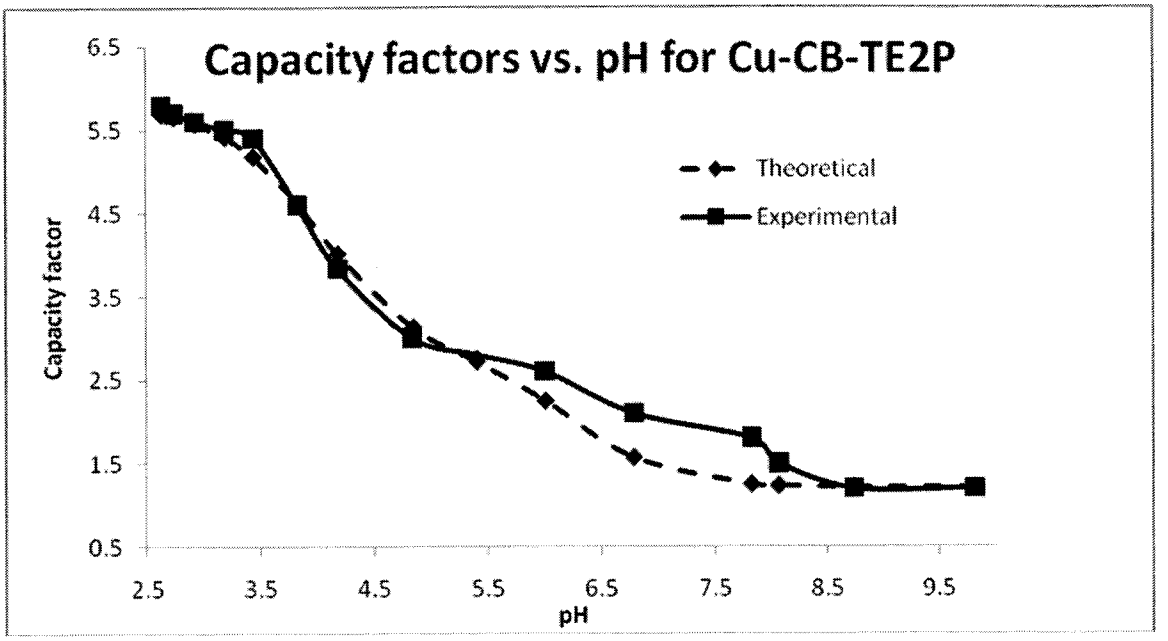


Figure 3.7. Plot of the capacity factor vs. pH on an Agilent C_{18} stationary phase.
Mobile Phase: 10 mM citrate at the indicated pH, $NaNO_3$ added to adjust ionic strength
Flow rate: 0.7 mL/min
Column: Agilent SB-C_{18} (50 × 4.6 mm; 3.5μm) Detection λ: 280 nm
Temperature: 25 °C Injection: 10 μL of 0.10 mg/mL of Cu-CB-TE2P
Experimental data shown in solid line and theoretical data shown in dashed line

pH	k'Theory	k' experimental
9.82	1.5	1.5
8.74	1.5	1.5
8.07	1.5	1.7
7.83	1.5	2.0
6.78	1.8	2.6
6.00	2.4	2.9
5.497	2.7	2.76
4.881	3.1	2.94
4.303	3.7	3.71
3.854	4.3	4.41
3.462	4.7	4.75
3.194	4.9	4.88
2.946	5.0	5.2
2.732	5.1	5.12
2.545	5.1	5.06

Table 3.3. The capacity factors for the Cu-CB-TE2P complex as a function of mobile phase pH on a Betabasic C_{18} stationary phase

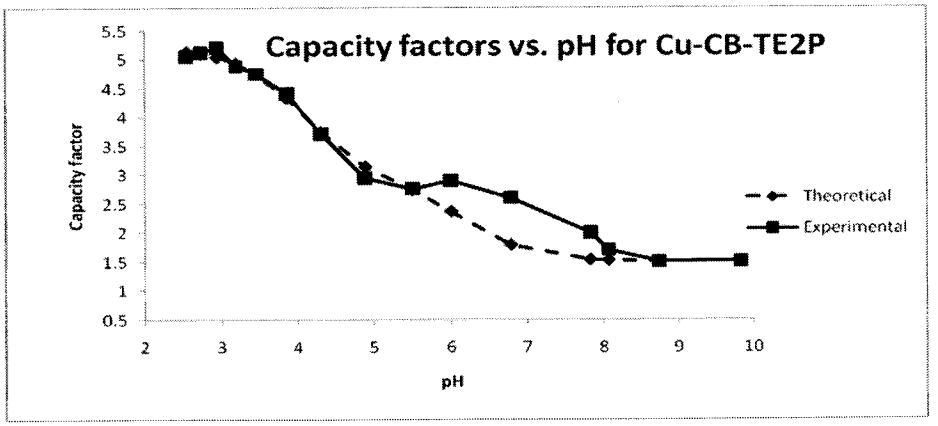


Figure 3.8. Plot of the capacity factor vs. pH on a Betabasic C_{18} stationary phase.
Mobile Phase: 10 mM citrate at the indicated pH, $NaNO_3$ added to adjust ionic strength
Flow rate: 2.0 mL/min
Column: Betabasic C_{18} (150 × 4.6 mm; 5μm) Detection λ: 280 nm
Temperature: 25 °C Injection: 10 μL of 0.10 mg/mL of Cu-CB-TE2P
Experimental data shown in solid lines and theoretical data shown in dashed line

The reason for performing the experiment on two different C_{18} stationary phases was to evaluate whether the stationary phase affected the determination of the pK_as for Cu-CB-TE2P. The differences between the stationary phases are shown in Table 3.4.

To obtain the pK_as of Cu-CB-TE2P, the chromatographic data shown in Figures 3.7 and 3.8 are fit to Equation 1.3. The Solver function on Excel was used to solve for the only unknowns, the two K_a values, in Equation 1.3.

$$k' = \frac{k'_0 + k'_{-1}\frac{K_{a1}}{[H^+]} + k'_{-2}\frac{K_{a1}K_{a2}}{[H^+]^2}}{1 + \frac{K_{a1}}{[H^+]} + \frac{K_{a1}K_{a2}}{[H^+]^2}} \quad \textbf{(Equation 1.3)}$$

k' measured capacity factor
k'_0 capacity factor for neutral form
k'_{-1} capacity factor for -1 charged form
k'_{-2} capacity factor for -2 charged form
K_{a1} 1st dissociation constant
K_{a2} 2nd dissociation constant
$[H^+]$ concentration of H_3O^+

The value for the hydronium ion concentration is obtained by measuring the pH of the mobile phase. The capacity factors for the various forms of Cu-CB-TE2P are determined chromatographically. The assumption is made that the lower pK_a of the complex is 2 or higher. If the pK_a is lower than 2, it would be almost impossible to measure it with the C_{18} stationary phase due to the possibility of hydrolysis of the silane ligand. If the pK_a was 2 then the pH of the mobile phase would need to be at least 1 and stability studies have shown a pH of 1.5 to be the lower limit for the Betabasic C_{18} stationary phase.[63]

Table 3.4 gives a summary of the capacity factors for the different ionic species of Cu-CB-TE2P. At lower pH, approximately 2.2 to 2.5, k'_0, the capacity factor for the undissociated form of Cu-CB-TE2P was determined experimentally. The capacity factor k'_{-2} for the fully dissociated species (-2 charged) was determined at a pH of approximately

8.7 to 9.8. The capacity factor k'_{-1} for the amphiprotic species (-1 charged) was assigned as the one measured for the Cu-CB-TE2P at pH 5.4, where the predominant species in solution (over 95%) is the amphiprotic form of the complex.

Capacity factors for the different species of Cu-CB-TE2P	Mobile phase pH	Capacity factor on Agilent C_{18} stationary phase	Capacity factor on Betabasic C_{18} stationary phase
k'_0	2.7	5.7	5.1
k'_{-1}	5.4	2.8	2.8
k'_{-2}	9.8	1.2	1.5

Table 3.4. Values of the capacity factors for different ionic species of Cu-CB-TE2P

The capacity factors of the complex under several mobile phase conditions were measured for an aqueous sample containing 0.10 mg/mL of Cu-CB-TE2P. The ionic strength is usually controlled for both potentiometric and spectrophotometric titrations. The pK_a values reported in the literature are typically measured at a concentration of 0.1 M sodium chloride or sodium nitrate, which maintains the ionic strength at 0.1. In an effort to allow a comparison of the chromatographic results with potentiometric results for similar compounds reported in the literature varying amounts of $NaNO_3$ were added to the mobile phase (always making sure the ionic strength of the solution was at 0.1). Mobile phases which did not contain an organic modifier such as methanol or acetonitrile were used to measure the two K_a values for Cu-CB-TE2P.

The experimental dissociation constants were estimated by performing a non-linear fit using the Excel Solver function. The K_a values obtained were entered into Equation 1.3 and the fits shown in the dashed line represent the predicted capacity factor as a function of mobile phase pH. A comparison of the predicted and experimental

capacity factors is shown in Figure 3.7 and 3.8. There is reasonable agreement between the predicted capacity factors (using the K_as from the fit) and the experimentally obtainable values for Cu-CB-TE2P. The overall shapes of both curves are consistent with the pK_a values of 4.02 and 6.25 as estimated on the Agilent SB-C_{18} column. It is expected at a pH which is one unit less than the pK_{a1} (4.02-1 i.e. 3.02) the dominant species (about 90% of the complex) is going to be undissociated. The capacity factor for the Cu-CB-TE2P complex is expected to be almost constant for mobile phases that have a pH below 3.05 and the experimental values are consistent with this expectation. The capacity factors at a mobile phase pH of 2.54 and 2.73 are 5.06 and 5.12, respectively, on the Betabasic C_{18} stationary phase. The capacity factors at a mobile phase pH of 2.58 and 2.74 are 5.74 and 5.79, respectively, on the Agilent SB C_{18} stationary phase. It is expected at a pH which is one unit higher than the pK_{a2} (6.25 + 1 i.e. 7.25) the dominant species (about 90% of the complex) will be fully dissociated. The capacity factors at a pH of 8.74 and 9.82 for the fully dissociated form of Cu-CB-TE2P are both 1.20 on the Agilent SB C_{18} column. The capacity factors at a pH of 8.74 and 9.82 for the fully dissociated form of Cu-CB-TE2P are both 1.53 on the Betabasic C_{18} column. At the present time there has not been a potentiometric evaluation of the pK_as for this complex, however data in the literature for similar compounds suggests that the values obtained are reasonable.[74-76] The Cu-TE2P has published pK_a values of 5.27 and 6.39 determined potentiometrically.[75] The difference between the complexes arises from the fact that the ligand used to synthesize the Cu-CB-TE2P complex contains an ethylene bridge between two non adjacent nitrogens. A comparison of the retention for Cu-CB-TE2P on two different C_{18} stationary phases shows that while there are differences in the values of the

capacity factors, these differences do not significantly affect the prediction of the K_as for the complex. The values obtained from the two stationary phase for the both pK_as of Cu-CB-TE2P are the within 5% of each other. The pKa values obtained for each stationary phase are shown in Table 3.5.

Stationary phase	Estimated pK_{a1}	Estimated pK_{a2}
Agilent C_{18}	4.02	6.25
Betabasic C_{18}	4.07	6.18

Table 3.5. Estimation of pK_a values for Cu-CB-TE2P on two different C_{18} stationary phases

3.3.3 Attempted estimation of pK_as of Cu-CB-TE2P using the porous graphitic carbon and modified silica hydride stationary phases

Additional experiments were performed using a PGC and modified silica hydride stationary phase to investigate whether or not they are suitable for use in estimating the pK_as of Cu-CB-TE2P. As indicated previously, the PGC stationary phase provides not only hydrophobic interactions but also other interactions such as electronic and polar-type interactions. These types of interactions had been previously reported by Lim and coworkers.[68] The pH of the mobile phase was adjusted from 2.54 to 6.00. The effect of pH on the capacity factor for Cu-CB-TE2P is presented in Figure 3.9. The results suggest that other interactions besides hydrophobic interactions are taking place on the PGC stationary phase. On both C_{18} columns the dissociated form of the Cu-CB-TE2P complex was retained less than the undissociated forms as it can be seen from the capacity factors given in Tables 3.2 and 3.3. The data show that there is an increase in the capacity factor when small amounts of the amphiprotic species are in solution for the

PGC stationary phase. The capacity factors decrease once the undissociated species is converted to the amphiprotic species. It is clear from the data that additional interactions besides hydrophobic interactions are likely responsible for the retention of Cu-CB-TE2P on the PGC stationary phase. Due to additional interactions it is not possible to accurately estimate the two K_a values of Cu-CB-TE2P using the PGC stationary phase.

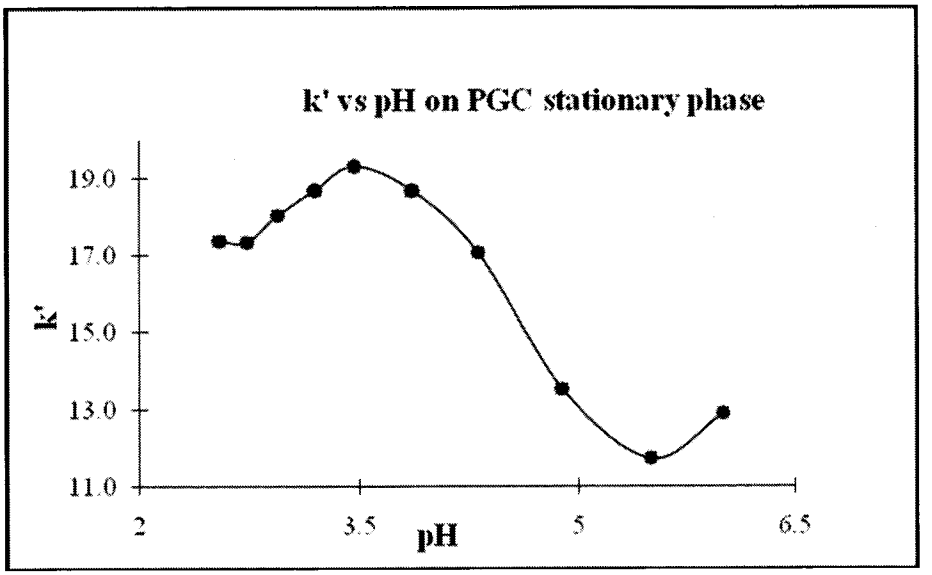


Figure 3.9. Plot of the capacity factor for Cu-CB-TE2P vs. pH on a porous graphitic carbon stationary phase using 10 mM citrate buffer with $NaNO_3$ added to adjust ionic strength as the mobile phase

The modified silica hydride stationary phase is a phase that can be used under both reversed and normal phase conditions. In order for an accurate comparison of the capacity factors for Cu-CB-TE2P among the different stationary phases utilized, the complex was eluted using reversed phase conditions. A 10 mM citrate buffer solution containing varying amounts of sodium nitrate to adjust the ionic strength was used to elute Cu-CB-TE2P. This stationary phase provided enhanced retention for the Cu-CB-TE2P complex with capacity factors as high as 50. The peak shape exhibits tailing as the

mobile phase does not contain methanol (Figure 3.6). The addition of methanol to the mobile phase significantly improved the peak shape. The pH of the mobile phase was adjusted to observe the effect of pH on the capacity factor for Cu-CB-TE2P. Variations in the capacity factor for Cu-CB-TE2P as a function of pH can be seen in Figure 3.10. The enhanced retention on the modified silica hydride stationary phase for this complex is a result of additional non hydrophobic interactions with the stationary phase. One of the possible retention mechanisms when using a modified silica hydride stationary phase is ion exchange. As shown in Figure 3.10, the amphiprotic form of the complex was retained longer than the undissociated form of the Cu-CB-TE2P. The capacity factor for the complex on this stationary phase shows an immediate jump when the pH is increased instead of the gradual changes that were observed on the C_{18} and PGC stationary phases.

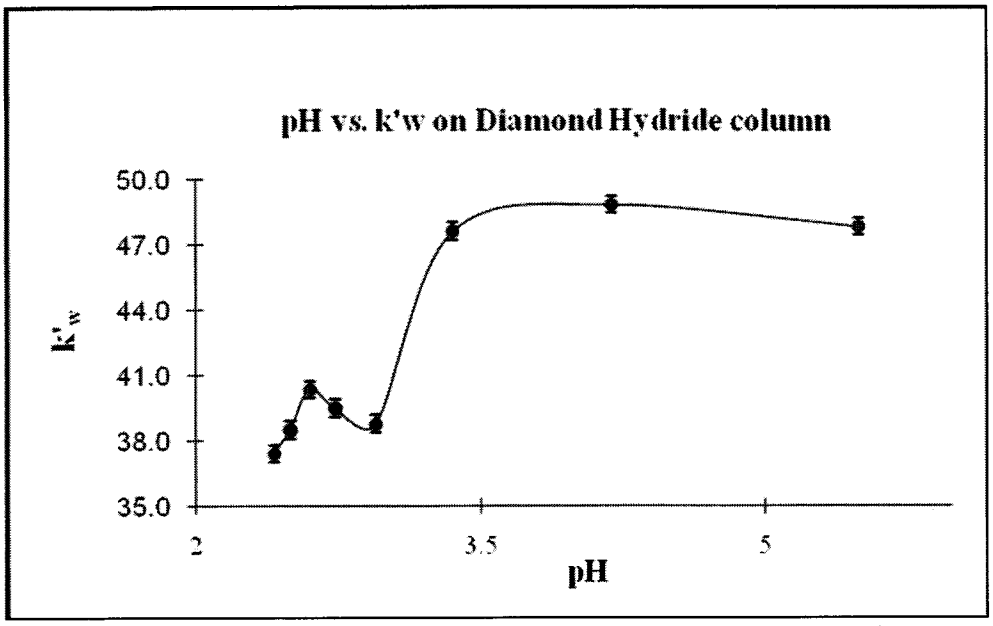


Figure 3.10. Plot of the capacity factor for Cu-CB-TE2P vs. pH on a diamond hydride stationary phase using 10 mM citrate buffer with $NaNO_3$ added to adjust ionic strength as the mobile phase

3.3.4 Estimation of the lipophilicity parameters for the Cu-CB-TE2P, Cu-CB-TE1A1P, Cu-CB-TE2P^{OEt}, Cu-CB-DO2P^{OEt}

The lipophilicity of a compound is related to its partitioning (P) between an organic and an aqueous solution. Typically the compound's octanol/water partition coefficient (P), as discussed in Chapter I is used as an indicator for lipophilicity. For many years log P (used instead of P for ease of reporting) has been considered as a useful measure of the lipophilicity of an analyte. Log P is important when synthesizing molecules that have potential *in vivo* applications. The lipophilicity of the molecules and variations to it are likely to affect its biodistribution and their permeability through membranes. In recent years log k'_w (k'_w is the capacity factor of an analyte measured using a 100 % aqueous mobile phase and typically a C_{18} stationary phase) has been used as a an indicator of lipophilicity.[33,63] It has been shown that there is good correlation

between log k'_w and log P prompting the substitution of log P values with log k'_w values. The capacity factor is extrapolated, to the 100 % aqueous mobile phase conditions based on chromatographic data acquired on mobile phases containing various amounts of organic modifier. The reason for extrapolating is that sometimes it is difficult to elute a compound with a mobile phase containing no organic modifier. However, it has also been shown that there is less correlation between the log P values and the extrapolated log k'_w .[63] The complexes shown in Table 3.6 can all be eluted with a 100% aqueous mobile phase on a C_{18} stationary phase. The measured capacity factors and log k'_w values are given in Table 3.7. The mobile phase consisted of 10 mM citrate buffer with a pH of 2.5. A pH of 2.5 was chosen for the mobile phase so that the Cu-CB-TE2P which is ionazible under the pH range 3 – 9 is in the undissociated form. The Cu-CB-TE1A1P is undissociated because it is ionazible in the pH range from 5 – 9.

Compound	**Capacity Factor**	**log k'_w**
Cu-CB-DO2A*	1.3	0.11
Cu-CB-TE2A*	5.9	0.77
Cu-CB-TE2P	5.2	0.72
Cu-CB-TE1A1P	8.5	0.93
Cu-CB-DO2P^{OEt}	56	1.75
Cu-CB-TE2P^{OEt}	155	2.19

Table 3.6. Experimentally-determined capacity factors and log k'_w for copper complexes on Betabasic C_{18} stationary phase.
Mobile Phase: 10 mM citrate at pH 2.5
Flow rate: 1.7 mL/min
* determined by Shen on Betabasic C_{18} using a mobile phase of 30 mM citrate at pH 2.5[35]

In an effort to investigate whether there is a bias from the extrapolation of the capacity factor, a study was undertaken. The complexes shown in Table 3.8 except Cu-CB-TE1A1P were eluted using mobile phases containing methanol. The content of methanol in the mobile phase varied from 1 percent to 10 percent. The extrapolation was done by performing a linear fit (log k' vs. % organic modifier) on the chromatographic data obtained (shown in Figure 3.8).

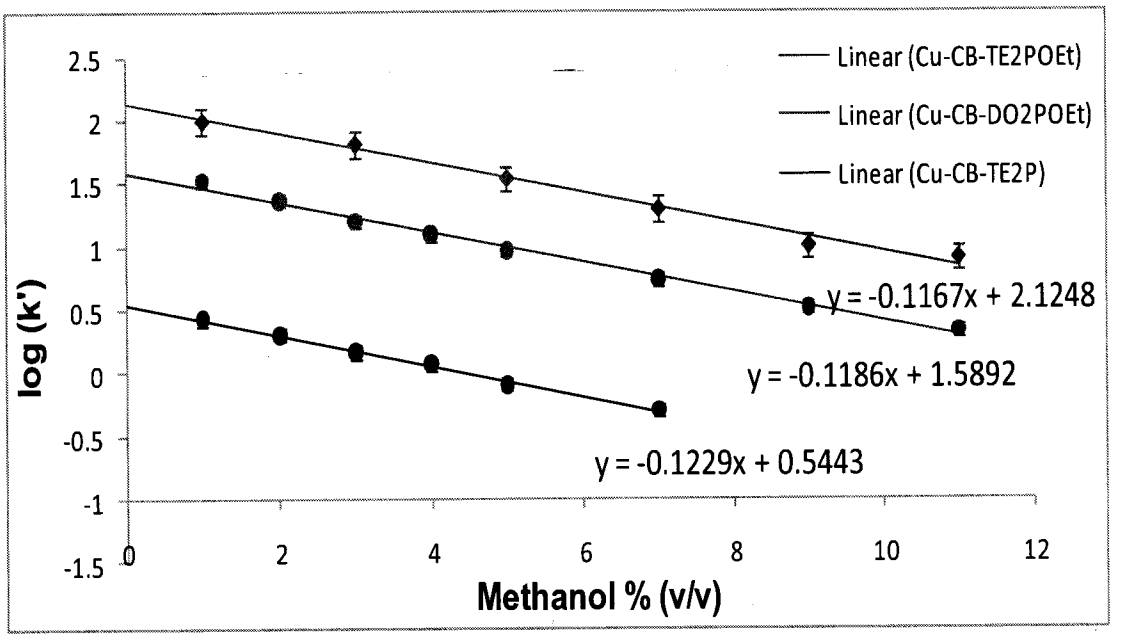


Figure 3.11. Plot of logk' for Cu-CB-DO2P^{OEt}, Cu-CB-TE2P^{OEt}, and Cu-CB-TE2P on the Betabasic C_{18} column

A comparison of the extrapolated values vs. the measured values for the capacity factors of the five complexes is given on Table 3.7. Though the data fitted were extrapolation was done for data points very close to 100% aqueous mobile phase, a clear distinction between the extrapolated and the experimentally determined capacity factors at 0% methanol was observed. The differences in capacity factors are larger for the more strongly retained complexes such as Cu-CB-DO2P^{OEt} and Cu-CB-TE2P^{OEt}. The results

support previous reports that it is better to measure the capacity factor with an aqueous mobile phase as there will likely be some error associated with extrapolated values. The values for the complexes studied cannot be compared with the actual log P values as there have been no reports on the measurement of these values by other methods. However the chromatographic data is important as it has clearly established trends for the lipophilicity of the copper complexes shown in Table 3.9.

Compound	**Capacity Factor (0% methanol)**	**Capacity Factor (extrapolated to 0% methanol)**	**Percent Difference between the Capacity factors**
Cu-CB-DO2A*	1.3	1.2	7.7
Cu-CB-TE2A*	5.9	5.0	15.3
Cu-CB-TE2P	5.2	3.5	32.7
Cu-CB-DO2P^{OEt}	56	39	30.4
Cu-CB-TE2P^{OEt}	155	132	14.8

Table 3.7. Extrapolated capacity factors for copper complexes.

* determined by Shen on Betabasic C_{18} using a mobile phase of 30 mM citrate at pH 2.5[35]

3.4 Conclusion

The chromatographic behavior of four recently synthesized methyl phosphonate pendant armed copper complexes has been investigated. Ring size plays an important role in elution order in RP-HPLC. In addition the functionality of the pendant arms influences the chromatographic behavior of the complexes. The functionality of the

pendant arms is the most important factor in determining the elution order and impacts the lipophilicity parameters of the complexes.

The PGC and silica hydride based stationary phases provide additional interactions with the complexes resulting in increased retention. It is possible that both electronic and polar type interactions occur between the copper complexes studied and the PGC stationary phase. An ion exchange mechanism and increased selectivity for phosphonate containing complexes is the reason for increased retention of these complexes on the silica hydride based stationary phase. pH affects the retention behavior of Cu-CB-TE2P and Cu-CB-TE1A1P because these complexes are ionazible in the pH range 3.0 to 7.5 and 5.5 to 9.5, respectively. Cu-CB-TE2P^{OEt} and Cu-CB-DO2P^{OEt} are unaffected by pH.

The chromatographic behavior of Cu-CB-TE2P is clearly pH dependent on the three stationary phases evaluated. Experimental results show that a C_{18} stationary phase provides for the best estimation of the pK_as for Cu-CB-TE2P. The determination of the pK_a values for Cu-CB-TE2P at 4.05 and 6.45 could be used to improve complexation conditions and predict *in vivo* behavior. The complexation reactions for Cu-CB-TE2P can be run at a pH of 3 or lower where the complex will be mostly in the neutral form. The capacity factors for the Cu-CB-TE2P, Cu-CB-TE1A1P , Cu-CB-DO2P^{OEt}, and Cu-CB-TE2P^{OEt} complexes with no organic modifier were measured, providing insights into the lipophilicity of these compounds. The measurement of the lipophilicity factors also allows for the comparison of these complexes with previously studied complexes such as Cu-CB-DO2A and Cu-CB-TE2A. Cu-CB-DO2A is the least lipophilic compound of the group. Cu-CB-DO2A has the smallest capacity factor on the three stationary phases. The

neutral form of Cu-CB-TE2P and Cu-CB-TE2A have similar lipophilic behavior based on the capacity factors obtained on the C18 stationary phase. The undissociated form of Cu-CB-TE1A1P is more lipophilic than both Cu-CB-TE2A and the undissociated form of Cu-CB-TE2P. Cu-CB-DO2P^{OEt} and Cu-CB-TE2P^{OEt} are the most lipophilic compounds in this group due to their ethyl ester arms.

CHAPTER IV

ENANTIOSEPARATION OF SELECTED POLYCYCLIC TETRAMINES USING ISOCRATIC HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

4.1 Introduction

Tetraazamacrocycles, such as cyclam, are polyamine ligands that have been shown to provide stable metal complexes. Polyamine ligands are of current interest due to their potential application as cancer diagnostic and therapeutic agents when complexed to radioactive metals.[7-9,33] An example of a polyamine ligand that has been used as a diagnostic agent is DTPA. In 1994 FDA approved ^{111}In-DTPA-octreotide (Octreoscan) as a diagnostic agent for neuroendocrine tumors. CB-TE2A, a cross-bridged polyamine ligand, has been shown to form stable metal complexes which are desirable for *in vivo* applications.[79] Cross-bridged cyclams are chiral and are synthesized as racemates. It is important to be able to separate and study the complexes of enantiomers of such ligands as these enantiomeric complexes may have different activities.

High performance liquid chromatographic methods are widely used to carry out chiral separations for pharmaceuticals. A recent review in LCGC indicated that over 10 percent of total separations being carried out are chiral separations.[1] There is an array of commercial chiral stationary phases used to provide enantioseparation of chiral amines. The most commonly used chiral stationary phases are based on polysaccharides, proteins,

antibiotics, Pirkle complexes and cyclodextrins.[81-86] Based on literature reviews, the majority of enantioseparations for polyamines have been achieved on polysaccharide stationary phases. The most popular mobile phases used for these enantioseparations are hexane/alcohol (ethanol or propanol). Other weak solvents such as pentane or heptanes have also been used instead of hexane to produce the separations.[87] The addition of acidic modifiers such as ethanesulfonic acid (ESA) or trifluoro acetic acid has been shown to improve peak shape and resolution.[89-92] In some cases including basic modifiers such as diethylamine (DEA) or triethylamine in the mobile phase has resulted in resolution when the acidic modifiers do not provide adequate resolution.[92,93-97]

Pirkle et al. established criteria which describe how a chiral stationary phase distinguishes between the two enantiomers.[98] These criteria state that "chiral recognition requires a minimum of three simultaneous interactions between the chiral stationary phase and at least one of the enantiomers, with at least one of these interactions being stereochemically dependent".[47,98]

In this study three polysaccharide stationary phases were used to obtain chiral separation of cross-bridged tetraazamacrocyclic compounds. The functional groups of each of the stationary phases are shown in Figures 4.1a-c. One of the stationary phases used, Chiralcel OD-R, is based on modified cellulose units. Cellulose has a mostly linear but highly ordered structure consisting of D-(+)-glucose units which form helical structures. The helical structures are believed to be responsible for discriminating between the enantiomers. The other two stationary phases, Chiralpak AD and Chiralpak IB, utilize modified amylose units.

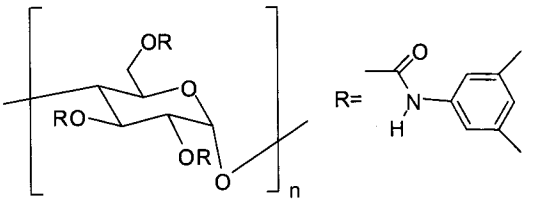


Figure 4.1.a. Chiralpak AD stationary phase (amylose derivative coated on silica).

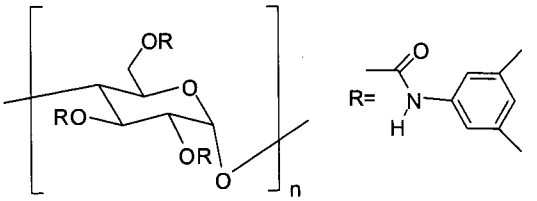


Figure 4.1.b. Chiralpak IB stationary phase (amylose derivative immobilized on silica).

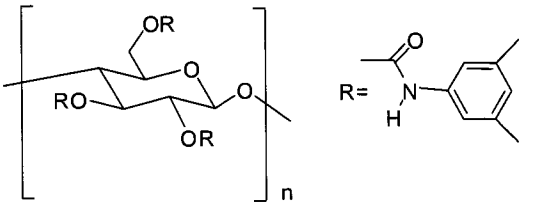


Figure 4.1.c. Chiralcel OD-R stationary phase (cellulose derivative coated on silica).

The Chiralcel OD-R stationary phase is cellulose tris(3, 5-dimethylphenylcarbamate) coated on 10μm silica. This stationary phase allows for a larger selection of mobile phases, including water, to achieve enantioseparation.[96-103] Computational studies with a cellulose tris(phenylcarbamate) chiral stationary phase and enantiomers of trans-stilbene oxide and trans-1,2 diphenylcyclopropane demonstrated that there are several interactions that could contribute to chiral recognition

of enantiomers.[101] Among these are hydrogen bonding, π-π and van der Waals interactions.

Derivatized amyloses are another polysaccharides that have also been successfully used as a stationary phase. For example, the Chiralpak AD stationary phase is an amylose tris (3, 5-dimethylphenylcarbamate) coated on 10μm silica. Unlike Chiralcel OD-R this stationary phase can only be used under normal phase conditions. The Chiralpak IB stationary phase is amylose tris(3, 5-dimethylphenylcarbamate) immobilized on 10μm silica. This stationary phase is typically used under normal phase conditions but tolerates a wider range of modifiers compared to Chiralpak AD. The 3, 5-dimethylphenylcarbamate is the same unit that is used to modify the cellulose in the Chiralcel OD-R stationary phase. Therefore it is reasonable to expect some of the same interactions will take place between the analytes and these stationary phases.

However, there are differences between the chiral stationary phases being used resulting in different chromatographic behavior for each of the enantiomeric pairs. The main difference among the chiral stationary phases being employed for this study is the type of modified polysaccharide coated on to the silica support material. Chiralpak AD acts solely as a normal stationary phase unless methanol has been added to mobile phases used with this stationary phase. It is known that the addition of methanol to the mobile phase causes irreversible changes in the Chiralpak AD stationary phase. After being exposed to methanol, this stationary phase should be dedicated to reversed phase elution conditions thereby limiting its application.[94] The Chiralcel OD-R can be used in both reversed phase and normal phase modes. The manufacturer's suggested organic mobile phase modifiers are another difference between these stationary phases. For example, the

use of acetonitrile and methanol containing mobile phase is not recommended for the Chiralpak AD stationary phase while these solvents are allowed when using Chiralcel OD-R. Another difference among the stationary phases is that the Chiralpak IB stationary phase is immobilized rather than coated such as the Chiralpak AD or Chiralcel OD-R.

Chromatographic studies were performed to investigate conditions which provide HPLC separation of the enantiomers of two cross-bridged tetraazamacrocycles and one precursor. One of the enantiomeric pairs successfully resolved is dibenzocyclam bisaminal, which is a precursor that upon further reactions results in a variety of cross-bridged ligands. The structures of CB-cyclam and dimethyl dibenzo-CB-cyclam, the other two ligands separated are presented in Figure 1.1.

Enantioselective chromatographic methods can be used for the determination of enantiomeric ratios for mixtures of enantiomers. The racemization barrier ($\Delta G^{\ddagger}$) refers to the activation energy required for conversion of one enantiomer to the racemate. The $\Delta G^{\ddagger}$ associated with the racemization of related ligands to CB-cyclam, such as dimethyl CB-cyclam, have been investigated by Weisman and Hines.[11] Based on the results for dimethyl CB-cyclam it was postulated that CB-cyclam would be resolvable under room temperature conditions. In order to obtain $\Delta G^{\ddagger}$ of racemization for CB-cyclam a kinetic experiment measuring the conversion of enantiopure compound to racemate as a function of time was performed. In order to speed up the racemization a temperature higher than 25°C was chosen. A temperature of 82 °C was used so racemization of enantiopure (*S,S*) CB-cyclam occurred within a reasonable period of time. The $\Delta G^{\ddagger}$ of racemization was determined by NMR and is reported to be 31.29±0.05 kcal/mol.[11] In this work the established separation conditions for CB-cyclam allowed for the measurement of the

racemization barrier. The value measured chromatographically was compared with the published value obtained using NMR.

4.2 Experimental

4.2.1 Reagents

HPLC-grade methanol and absolute ethanol were purchased from Pharmco AAPER (Brookfield, CT, USA). Acetonitrile, hexanes and isopropanol, all HPLC-grade, were purchased from Fisher Scientific (Fairlawn, NJ, USA). The DI water used for the preparation of the standards and the eluents was obtained from a MilliQ water system. All mobile phases were filtered through a 0.45 μm nylon filter (Whatman, OR, USA) prior to use. ACS grade toluene was obtained from EMD Chemicals Inc. Diethanol amine and ethane sulfonic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Diethyl amine 99.5 % pure was purchased from Fluka a Sigma-Aldrich subsidiary (St. Louis, MO, USA).

4.2.2 Apparatus

Chromatographic separations were performed using a Varian 9010 pump fitted with a Rheodyne 7125 injector (Cotati, CA, USA) and a 10 μL injection loop. For the chromatographic separations using normal phase solvents a Waters 600 pump (Milford, MA, USA) fitted with a Rheodyne 7125 injector (Cotati, CA, USA) and a 10 μL injection loop was used. UV detection for both pumps was performed using a Waters 486E UV-Vis absorbance detector (Milford, MA, USA). The detector was set at 237 nm to monitor CB-cyclam or 268 nm to monitor dibenzobisaminal and dimethyl dibenzo CB-cyclam. The detector was interfaced to a laboratory computer data system or a Kipp and Zonen

BD41 chart recorder. The columns were held at the indicated temperature using a column heater (Jones Chromatography, Hengoed, UK) or to maintain 0° C immersed in an ice bath. The retention properties of the analytes were investigated using three chiral stationary phases: (1) Chiralpak AD (250 × 4.6 mm; 10μm; Daicel Technologies, Exton, PA, USA), (2) Chiralcel OD-R (250 × 4.6 mm; 10μm; Daicel Technologies, Exton, PA, USA), and (3) Chiralpak IB (250 × 4.6 mm; 10μm; Daicel Technologies, Exton, PA, USA). A Polystat Circulating Constant Temperature bath (Cole Parmer, Vernon Hills, IL, USA.) was used to heat the enantiomer to the desired temperature for the racemization experiment. A refrigerator was used to store the aliquots of sample until the run was complete.

4.2.3 Chromatographic Conditions

Prior to use, each chromatographic column was equilibrated for at least 30 column volumes with the mobile phase to be used for the experiment. The mobile phase flow rate was adjusted for each column (0.5 mL/min to 1.2 mL/min) depending on mobile phase composition and temperature. The pressure generated was greater at high ethanol concentrations and low temperatures. The Chiralcel OD-R column was flushed with at least 30 column volumes of 70/30 methanol/acetonitrile (v/v) solution after daily use in the reversed phase mode. The Chiralcel OD-R column was flushed with at least 30 column volumes of 70/30 hexane/ethanol (v/v) solution after daily use in the normal phase-mode. The Chiralpak AD column was flushed with at least 30 column volumes of 70/30 hexane/ethanol (v/v) solution after daily use. The mobile phases not containing an additive were obtained by mixing the appropriate volumes of hexane and ethanol or other

modifiers as desired. In the case of the mobile phases with an additive, after combining the hexane and ethanol the flask was weighed and the appropriate amount of additive was added as a weight %. The most commonly used additive was diethyl amine. For a mobile phase containing diethylamine, injecting a solution of 15/85 (v/v) ethanol/hexane caused a disturbance to be observed in the chromatogram which was taken as the dead time (t_0) for the column. The selectivity factor, α, was calculated using the following equation 4.1

$$\alpha = \frac{k'_2}{k'_1} \textbf{ (Equation 4.1)}$$

where k'_2 is the capacity factor for the later eluting enantiomer and k'_1 is the capacity factor for the other enantiomer. The resolution, R_s, of the enantiomers was calculated using equation 4.2

$$R_s = 2 \times \frac{t_{R2} - t_{R1}}{w_1 + w_2} \textbf{ (Equation 4.2)}$$

where t_{R2} is the retention time for the later eluting enantiomer and t_{R1} is the retention time for the other enantiomer. The w_2 and w_1 are the peak widths for the later eluting enantiomer and the other enantiomer respectively.

4.2.4 Sample Preparation

CB-cyclam, dibenzocyclam bisaminal, and dimethyl dibenzo- cross-bridged-cyclam were prepared by the Weisman-Wong group according to published or unpublished methods.[28] The CB-cyclam and dibenzobisaminal samples for analysis by HPLC on the Chiralpak AD column were dissolved in a 15/85 ethanol/hexane (v/v) solution. The dibenzocyclam bisaminal sample was dissolved in ethanol for experiments using the Chiralcel OD-R column. The dimethyl dibenzo-CB-cyclam was dissolved in

acetonitrile or ethanol. The samples for the determination of the racemization rate were approximately 3 mg CB-cyclam each and dissolved in 15/85 (v/v) ethanol/hexane solution to an approximate concentration of 3.75 mg/mL.

4.3 Results and Discussion

4.3.1 Effects of mobile phase composition on retention of dibenzocyclam bisaminal, CB-cyclam and dimethyl dibenzo-CB-cyclam

Hexanes are commonly used in the mobile phase for the Chiralpak AD stationary phase and were chosen as the weak solvent for this work. Ethanol and 2-propanol were investigated for use as strong solvents to elute the enantiomers. One study in the literature examined the effect of protic and aprotic solvents on analyte retention on polysaccharide stationary phases such as Chiralpak AD and Chiralcel OD-R.[102] The results suggested that protic solvents such as ethanol can compete with the enantiomers for binding sites on the stationary phase thereby influencing enantiomeric resolution.[102]

The resolution of the CB-cyclam enantiomers was investigated on the Chiralpak AD stationary phase. Initially 2-propanol was investigated as the strong modifier in combination with hexane for attaining baseline enantioseparation of CB-cyclam. The chromatographic behavior of CB-cyclam for mobile phases containing varying amounts of 2-propanol was investigated. The mobile phases ranged from 10/90 (v/v) 2-propanol/hexane to as high as 40/60 (v/v) 2-propanol/hexane. The selectivity for the enantiomers was very poor and for certain mobile phase compositions only a single broad peak was observed when using Chiralpak AD as the stationary phase. Even the addition of diethylamine or diethanolamine at concentrations from 0.2 to 0.5 wt % to mobile

phases containing 2-propanol did not resolve the enantiomers on the Chiralpak AD stationary phase.

In an effort to obtain enantioseparation of CB-cyclam, ethanol was investigated as a strong mobile phase modifier. A mobile phase of 20/80 (v/v) ethanol/hexane mobile phase produced two broad tailing peaks, which were not fully resolved on the Chiralpak AD stationary phase. The amount of ethanol in the mobile was varied from 5/95 (v/v) ethanol/hexane to as high as 35/65 (v/v) ethanol/hexane without achieving baseline resolution. Adding DEA or diethanolamine to this mobile phase was found to improve the separation and afford baseline resolution. The highest resolution was achieved with a mobile phase of 15/85/2.0 (v/v/wt%) ethanol/hexane/diethanolamine (Figure 4.2).

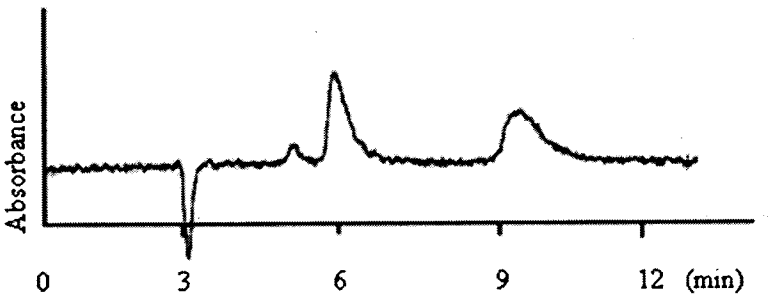


Figure 4.2. Enantioseparation of CB-cyclam 1mg/mL
Mobile Phase: 15/85/2 (v/v/wt %) ethanol/hexane/diethanolamine
Flow rate: 1.0 mL/min Column: Chiralpak AD (250 × 4.6 mm; 10μm)
Detection λ: 237 nm Temperature: 23 °C Injection: 10 μL of 1 mg/mL CB-cyclam

The second analyte of interest is dibenzocyclam bisaminal. The chromatographic behavior of dibenzocyclam bisaminal was investigated on all three stationary phases Chiralpak AD, Chiralpak IB and Chiralcel OD-R. It was determined that the enantiomers of dibenzocyclam bisaminal can be separated without the addition of any additive on both the Chiralpak AD and Chiralcel OD-R stationary phases, which is beneficial because the

pure enantiomers can be recovered easily. The enantioseparation of dibenzocyclam bisaminal on these stationary phases is shown on Figures 4.3a and b.

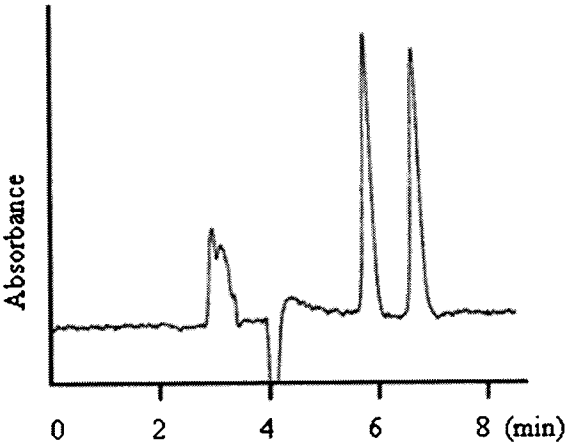


Figure 4.3.a. Enantioseparation of dibenzocyclam bisaminal injected at a concentration of 0.22 mg/mL
Mobile Phase: 25/75 (v/v) ethanol/hexane
Flow rate: 1.0 mL/min Column: Chiralpak AD (250 × 4.6 mm; 10μm)
Detection λ: 268 nm Temperature: 20 °C Injection: 10 μL

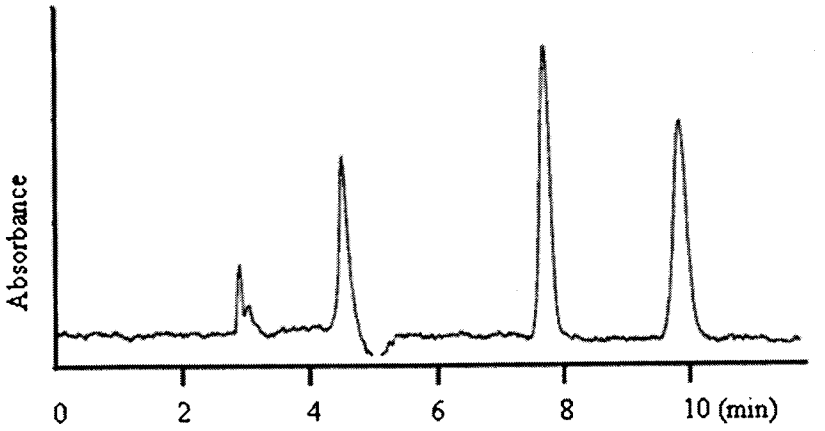


Figure 4.3.b. Enantioseparation of dibenzocyclam bisaminal injected at a concentration of 0.22 mg/mL
Mobile Phase: 25/75 (v/v) ethanol/hexane
Flow rate: 1.0 mL/min Column: Chiralcel OD-R (250 × 4.6 mm; 10μm)
Detection λ: 268 nm Temperature: 20 °C Injection: 10 μL

The resolution of the enantiomers of dibenzocyclam bisaminal could be further improved by addition of 0.15 wt% diethyl amine or 0.05 wt% ethanesulfonic acid to the mobile phase. The Chiralcel OD-R stationary phase was employed in reversed phase mode. Due to the hydrophobic nature of dibenzocyclam bisaminal it is possible to achieve reasonable retention of the enantiomers with low concentrations of water added to the mobile phase when using Chiralcel OD-R as the stationary phase. The dibenzocyclam bisaminal enantiomers have capacity factors of 4.1 and 5.5 respectively (Table 4.1) on the Chiralcel OD-R stationary phase when using a mobile phase of 90/10/0.3 methanol/water/diethylamine (v/v/wt %). The retention of the dibenzocyclam bisaminal enantiomers increased when the amount of methanol decreased, which is consistent with reversed phase behavior. Acetonitrile was used as the strong organic modifier instead of methanol to produce separations of similar resolution in a shorter time. For example, the capacity factors for 90/10/0.3 methanol/water/ DEA (v/v/wt %) are four times larger than those obtained for a 90/10/0.3 acetonitrile/water/ DEA (v/v/wt%) mobile phase on the Chiralcel OD-R stationary phase (Table 4.1 and 4.2). The difference between the capacity factors for the enantiomers of dibenzocyclam bisaminal is even greater for the 90/10/0.3 methanol/water/ DEA (v/v/wt %) to 90/10/0.3 acetonitrile/water/ DEA (v/v/wt%)

Mobile Phase ($MeOH/H_2O$)	Additive Diethyl amine	k'_1	k'_2	R_s
90/10	0.3	4.1	5.5	3.0
80/20	0.3	8.0	10.8	2.9

Table 4.1. Enantioseparation of dibenzocyclam bisaminal on Chiralcel OD-R using methanol as the strong solvent and diethylamine as the additive

Mobile Phase (ACN/H_2O)	Additive Diethyl amine	k'_1	k'_2	R_s
95/5	0.3	0.9	1.3	2.4
90/10	0.3	0.9	1.4	3.7
85/15	0.3	1.0	1.6	3.0
80/20	0.3	1.2	1.9	3.3

Table 4.2. Enantioseparation of dibenzocyclam bisaminal on Chiralcel OD-R using acetonitrile as the strong solvent and diethylamine as the additive

The separation of the enantiomers of dimethyl dibenzo- CB-cyclam was initially investigated on the Chiralcel OD-R stationary phase. The capacity factors for the enantiomers of this ligand were approximately 27 and 30 on Chiralcel OD-R when using a 85/15 acetonitrile/water (v/v) mobile phase. Variations in acetonitrile concentration did not provide baseline resolution of the enantiomers even though the capacity factors were affected as expected. Both enantiomers exhibited band broadening which could have been the result of interactions with the underlying silanols or due to the fact that the enantiomers had capacity factors over 30. Other weak solvents methanol and ethanol were used instead of water to reduce analysis time. The acetonitrile/methanol or acetonitrile/ethanol mobile phases did not improve the separation even though they did reduce the analysis time as expected. The addition of 0.3 wt% DEA to an acetonitrile/ethanol mobile phase improved resolution (Figure 4.4). Further improvement in peak shape was observed when the temperature was increased from 25 °C to 35 °C. Additional experiments demonstrated that baseline resolution can be achieved with either an acetonitrile/ethanol/DEA or acetonitrile/methanol/DEA mobile phase on the Chiralcel OD-R stationary phase at 35 °C.

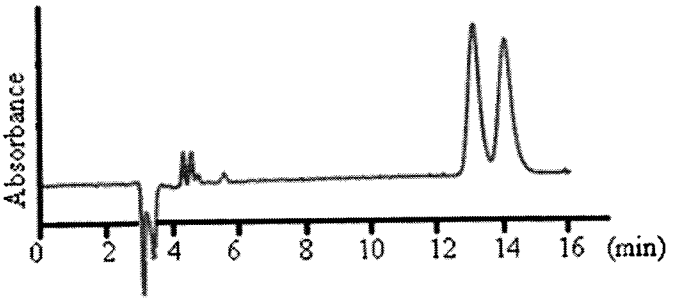


Figure 4.4. Enantioseparation of dimethyl dibenzo CB-cyclam injected at a concentration of 0.5 mg/mL
Mobile Phase: 4/96/0.3 (v/v/wt%) acetonitrile/ethanol/diethylamine
Flow rate: 1.0 mL/min Column: Chiralcel OD-R (250 × 4.6 mm; 10μm)
Detection λ: 268 nm Temperature: 35 °C Injection: 10 μL

Mobile Phase (ACN/EtOH)	Additive Diethyl amine	k'_1	k'_2	R_s
96/4	0.3	3.4	3.9	1.4
92/8	0.3	3.9	4.3	1.2
88/12	0.3	5.4	5.9	1.2
80/20	0.3	4.1	4.5	0.6
60/40	0.3	3.4	3.7	0.5

Table 4.3. The effect of mobile phase composition on the retention of dimethyl dibenzo CB-cyclam on the Chiralcel OD-R stationary phase

A very interesting chromatographic behavior was observed for the enantiomers of dimethyl dibenzo CB-cyclam when using mobile phases containing acetonitrile/ethanol for concentrations ranging from 96/4 to 60/40 (v/v). The data show that the retention for the enantiomers of dimethyl dibenzo- cross-bridged-cyclam is the greatest for 88/12/0.3 acetonitrile/ethanol/ diethylamine (v/v/wt %) mobile phase on the Chiralcel OD-R stationary phase. The capacity factor decreases with either increasing or decreasing acetonitrile concentration in the mobile phase as it can be seen on Table 4.3. Typically

acetonitrile would be considered the strong solvent and ethanol the weak solvent under reverse phase elution conditions. The fact that further decreases in acetonitrile concentration beyond 88/12/0.3 acetonitrile/ethanol/DEA (v/v/wt %) resulted in decreased capacity factors suggest that the stationary phase is behaving as a normal phase stationary phase. The data for the Chiralcel OD-R stationary phase suggest that the column can be used in either a normal phase mode or reversed phase mode depending on the properties of the enantiomers of the polycyclic tetraamines.

4.3.2 Evaluating the effect of the stationary phase type on enantioseparation

There are various interactions that are suggested in the literature which are responsible for the chiral recognition of the enantiomers by the polysaccharide stationary phase. The recognition interactions may involve hydrogen bonding, dipole-dipole interactions, π-π interactions and inclusion in a chiral groove.[95-98] It is very hard to predict the exact interactions responsible for chiral resolution of the cross-bridged tetraazamacrocycles being studied. The presence of the carbamate residue on the stationary phase may be important for inducing efficient chiral discrimination.[96] The carbonyl oxygen and the NH group of the carbamate residue on the stationary phase interact through hydrogen bonding with the polycyclic amines. Another major factor that could be responsible for the separation of the enantiomers in the case of dibenzocyclam bisaminal and dimethyl dibenzo- CB-cyclam are π-π interactions. Both of these analytes (dibenzocyclam bisaminal and dimethyl dibenzo- CB-cyclam) have aromatic functionalities, as does the tris(phenyl carbamate) group residue on the modified amylose/cellulose stationary phase. The presence of aromatic functionalities could be

responsible for enhanced interactions between the analytes (dibenzocyclam bisaminal and dimethyl dibenzo- cross-bridged-cyclam) and these stationary phases.

The effect of sample concentration on peak shape and retention times was investigated. Three samples having different CB-cyclam concentrations were analyzed in an effort to better understand these effects. The retention times of the enantiomers increased as the concentration decreased from 2.0 mg/mL to 1.0 mg/mL to 0.67 mg/mL. The capacity factors for the (S,S) CB-cyclam changed from 4.0 to 4.5 to 5.0 as the concentration decreased. The capacity factors for the (R,R) CB-cyclam changed from 5.6 to 6.0 to 6.4 respectively as the concentration decreased. Another reason for reducing the sample concentration was to minimize peak tailing. Peak tailing was calculated using equation 4.3 given below.[36]

$$T_f = \frac{W_{1.2back}}{W_{1/2front}} \quad \textbf{(Equation 4.3)}$$

The enantiomers of CB-cyclam exhibit peak tailing greater than 1.2 even at the 0.67mg/mL concentrations. The data suggest that there is overloading of the column but due to detection sensitivity issues more dilute samples of CB-cyclam can not be injected.

In order to further investigate overloading the behavior of dibenzocyclam bisaminal was investigated. The peak shape significantly improved as the concentration was decreased from 0.14 mg/mL to 0.07 mg/mL dibenzocyclam bisaminal is shown in Figures 4.5.a and b.

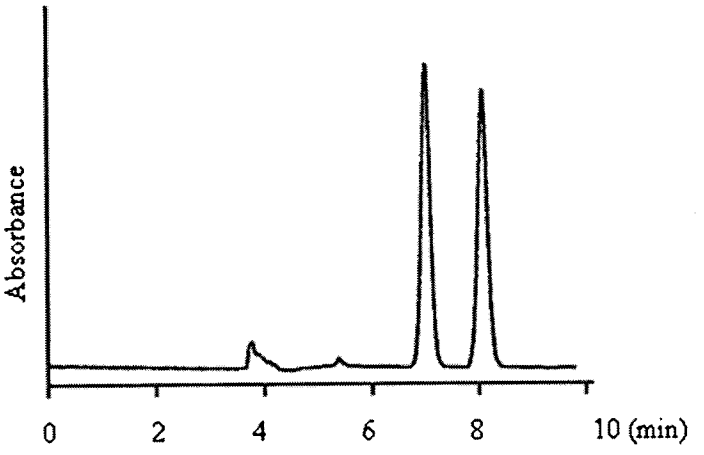


Figure 4.5.a. Chromatogram for the enantioseparation of dibenzocyclam bisaminal injected at a concentration of 0.07 mg/mL
Mobile Phase: 20/80/0.20 (v/v/wt %) ethanol/hexane/diethylamine
Flow rate: 1.0 mL/min Column: Chiralpak AD (250 × 4.6 mm; 10μm))
Detection λ: 268 nm Temperature: 25 °C Injection: 10 μL

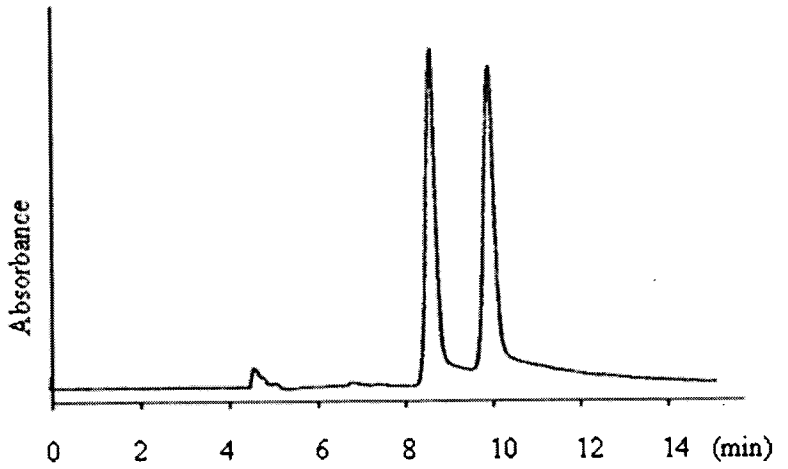


Figure 4.5.b. Chromatogram for the enantioseparation of dibenzocylam bisaminal injected at a concentration of 0.14 mg/mL
Mobile Phase: 20/80/0.20 (v/v/wt %) ethanol/hexane/diethylamine
Flow rate: 1.0 mL/min Column: Chiralpak AD (250 × 4.6 mm; 10μm))
Detection λ: 268 nm Temperature: 25 °C Injection: 10 μL

In addition to the Chiralpak AD a Chiralcel OD-R chiral stationary phase was investigated. The enantioseparation for CB-cyclam was attempted on the Chiralcel OD-R stationary phase using a mobile phase of 3/97 v/v methanol/water without any success. Various mobile phase compositions were evaluated; however, the enantiomers of

CB-cyclam were not retained on the Chiralcel OD-R stationary phase making it impossible to attain baseline resolution. If the same mobile phase as that utilized on Chiralpak AD 15/85/0.15 (v/v/wt%) ethanol/hexane/diethylamine is used no peaks are observed for 90 minutes. The attempted separation on the Chiralpak IB resulted in two peaks but no baseline resolution. Therefore for this analyte, CB-cyclam, the ideal stationary phase is the Chiralpak AD.

The separation of the enantiomers of dibenzocyclam bisaminal, was studied using all three stationary phases. Baseline resolution of its enantiomers was achieved on all stationary phases. Interestingly, it was found to be possible to resolve the dibenzocyclam bisaminal enantiomers with the same mobile phase composition [80/20/0.3 ethanol/hexane/DEA (v/v/wt %)] on both Chiralcel OD-R and Chiralcel AD stationary phases. The capacity factors and resolution are greater on the Chiralcel OD-R stationary phase for this mobile phase (1.1 and 1.5 with R_s 2.0 and 1.8 and 2.7 with R_s 4.5 on the Chiralpak AD and Chiralcel OD-R stationary phase respectively).

The separation of the enantiomers of dimethyl dibenzo CB-cyclam, was investigated solely on the Chiralcel OD-R stationary phase. The fact that acetonitrile can not be used on the Chiralpak AD limits the investigation of this compound to the Chiralcel OD-R stationary phase. It is probable that the enantiomers will stick to the stationary phase and take a long time to elute. This is demonstrated by the fact that the enantiomers of dimethyl dibenzo CB-cyclam could not be eluted in reasonable times when a methanol/water or ethanol/water mobile phase was utilized on the Chiralcel OD-R stationary phase. The solubility of the dimethyl dibenzo CB-cyclam enantiomers in hexane is not very high. Therefore the ability of hexane to elute the enantiomers from the

Chiralpak AD will be limited. The fact that amylose tris(3, 5-dimethylphenylcarbamate) is immobilized on the silica support (Chiralpak IB) instead of coated (Chiralpak AD) is seemingly the only difference between these stationary phases. The enantioseparation of dimethyl dibenzo CB-cyclam enantiomers was not attempted on the Chiralpak IB stationary phase either. Even though additives such as chloroform and THF are safe on this column, acetonitrile is not suitable as per recommendation of the manufacturer.

4.3.3 Evaluating the effects of adding diethylamine, diethanolamine, and ethanesulfonic acid to the mobile phase

It has been reported in the literature that the addition of modifiers such as DEA to the mobile phase greatly reduces peak tailing and improves column efficiency.[102] The addition of basic or acidic modifiers to the mobile phase may limit the interactions of the analyte with the underlying silanols.[99-101] The effect of DEA and diethanolamine on resolution and selectivity for the analytes were investigated. The effect of an acidic additive, ethanesulfonic acid ESA, was examined as well. The chiral separation may be affected by the presence of silanol interactions. However the increase in plate numbers resulted in improvements in the enantiomeric separation as shown by an increase in resolution. The amount of modifier present in the mobile phase and its effect on the chromatographic behavior for the enantiomers was evaluated.

For dibenzocyclam bisaminal, an enantioseparation was achieved even without the addition of diethylamine to the mobile phase when using Chiralpak AD or Chiralcel OD-R as the stationary phase. The difference in capacity factors for dibenzocyclam bisaminal between a mobile phase containing diethylamine and one that does not contain

the additive is given in Table 4.4. There is a slight increase in the retention of both enantiomers for dibenzocyclam bisaminal with the addition of diethylamine, however the resolution is still about the same. The peak shape and resolution show only minor variations with the addition of greater concentrations of DEA (0.2, 0.4, and 0.5 wt %).

Mobile Phase (Hexanes/EtOH)	Additive Diethyl amine	k'_1	k'_2	R_s
90/10	0	1.8	3.1	3.9
90/10	0.3	2.7	4.0	3.8

Table 4.4. Effect of diethylamine concentration on the enantioseparation of dibenzocyclam bisaminal when using hexane/ethanol mobile phase on the Chiralcel OD-R stationary phase

For dimethyl dibenzo-CB-cyclam the separation of the enantiomers was achieved only with the addition of diethylamine. In the absence of diethylamine there was increased band broadening and complete resolution of the peaks could not be achieved. The retention times were significantly longer in the absence of diethylamine, which is consistent with interactions of the analytes with the underlying silanols. The same chromatographic behavior was observed for the enantiomers of CB-cyclam. In the absence of an additive, the peaks were broad, possibly due to interactions with the silanols. The addition of DEA at 0.15 wt % to the 15/85 (v/v) ethanol/hexane mobile phase resulted in baseline resolution of the enantiomers.

In addition to DEA the effect of other basic mobile phase additives including diethanolamine was investigated. Diethanolamine had a similar effect as DEA when using the Chiralpak AD stationary phase. Various concentrations of diethanolamine and its effect on the resolution of CB-cylam enantiomers were investigated. The results in Table 4.5 show that the resolution of the enantiomers decreases with decreasing

diethanolamine concentration in the mobile phase. The loss in resolution could be a result of peak tailing.

Mobile Phase (Hexanes/Ethanol)	Diethanolamine concentration (wt%)	R_s
85/15	2	2.4
85/15	1	1.7
85/15	0.15	1.1

Table 4.5. Effect of diethanol amine concentration on the resolution of CB-cyclam when using hexane/ethanol mobile phase on the Chiralpak AD stationary phase (Temperature: 25 °C)

Acidic additives, such as ESA, have also been used to improve the resolution of polar analytes.[100-103] For example ESA lowers the apparent pH of the mobile phase, capping the silanols through protonation, while the basic additives such as DEA limit the effect of underlying silanols through an ion-pair mechanism.[101] Addition of ESA to the mobile phase resulted in increased retention for dibenzocyclam bisaminal when using Chiralpak AD as the stationary phase (Figure 4.6). A comparison of the capacity factors for identical compositions of hexane/ethanol clearly demonstrated that ESA provides increased retention compared to DEA on the Chiralpak AD stationary phase for dibenzocyclam bisaminal. The capacity factors for dibenzocyclam bisaminal with 15/85/0.2 ethanol/hexane/DEA (v/v/wt %) were 1.25 and 1.68 while for 15/85/0.05 ethanol/hexane/ESA were 16.29 and 20.29 respectively.

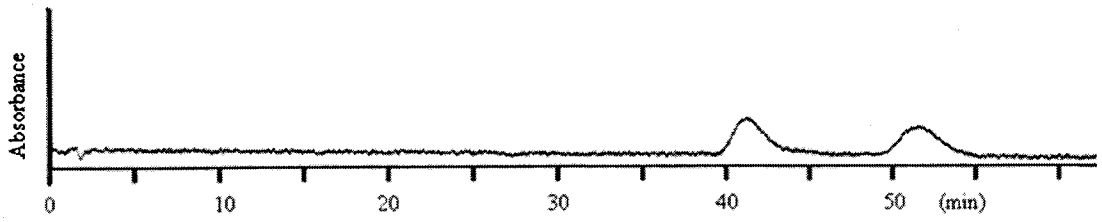


Figure 4.6. Chromatogram for the enantioseparation of dibenzocylam bisaminal using ethanesulfonic acid as an additive injected at a concentration of 0.50 mg/mL
Mobile Phase: 15/85/0.05 (v/v/wt %) ethanol/hexane/ ethane sulfonic acid
Flow rate: 1.0 mL/min Column: Chiralpak AD (250 × 4.6 mm; 10μm))
Detection λ: 268 nm Temperature: 25 °C Injection: 10 μL

Mobile phases containing ESA were not evaluated for CB-cyclam and dimethyl dibenzo-CB-cyclam as longer separation times are not desirable. The results obtained show that basic additives such as DEA and diethanolamine are more suitable for the enantioseparation of chiral polyamine ligands.

4.3.4 Evaluating the effect of temperature on the separation of the enantiomers of CB-cyclam, dibenzocyclam bisaminal and dimethyl dibenzo-CB-cyclam

Reports in the literature suggest that in most cases increasing the column temperature will result in decreased retention and may result in improved separation of the enantiomers.[99] All three analytes used in this study, CB-cyclam, dibenzocyclam bisaminal and dimethyl dibenzo-CB-cyclam, were found to behave in the same way: their retention times decreased when the temperature of the Chiralcel OD-R and Chiralpak AD stationary phases increased over the 20°-40° C and 0°-30° C temperature ranges respectively. At lower temperatures there was an increase in selectivity for the CB-cyclam enantiomers but the peaks showed tailing, resulting in a loss of resolution. On the other hand, at higher temperatures resolution was lost as selectivity for the

enantiomers decreased possibly due to smaller selectivity of the Chiralpak AD stationary phase for this analyte. The results showed that the best temperature for the separation of the CB-cyclam enantiomers on the Chiralpak AD stationary phase is at 22 °C. The best resolution for dimethyl dibenzo-CB-cyclam was achieved at 35° C on the Chiralcel OD-R stationary phase. The other analyte of interest, dibenzocyclam bisaminal was well resolved over the range of 20 to 35° C on the Chiralcel OD-R. There were only slight changes in selectivity from around 1.9 to 1.7 as the temperature was raised from 20° C to 35° C. The capacity factors for the dibenzocyclam bisaminal enantiomers are presented in Table 4.6. As expected, the increase in temperature resulted in reduced capacity factors and a shorter analysis time.

T (°C)	k'_1	k'_2	R_s	Selectivity
20	2.2	4.1	4.7	1.9
25	1.7	3.0	4.4	1.8
30	1.5	2.6	4.1	1.8
35	1.3	2.2	3.7	1.7

Table 4.6. Effect of temperature on the enantioseparation of dibenzobisaminal cyclam when using a 15/85/0.3 (v/v/wt%) ethanol/hexane/diethylamine mobile phase on the Chiralcel OD-R

Based on the results obtained for all three analytes, peak tailing and band broadening improved as the temperature increased. The theoretical plate numbers improved with increases in temperature and this resulted in improved resolution for dimethyl dibenzo-CB-cyclam. On the other hand, for CB-cyclam there was an increase in plate numbers and lower selectivity. The lost selectivity for the enantiomers of CB-cyclam resulted in lower resolution.

4.3.5 Determination of $\Delta G^{\ddagger}$ of racemization of CB-cyclam

One of the goals of developing chromatographic conditions which provide enantiomeric separation of the cross-bridged tetraazamacrocycles was to evaluate the enantiomerization barrier. The separation for the determination of $\Delta G^{\ddagger}$ for CB-cyclam was carried out using a 15/85/0.15 (v/v/wt %) ethanol/hexane/DEA mobile phase on the Chiralpak AD stationary phase. In order to determine $\Delta G^{\ddagger}$ it is easier to start with one enantiomer and measure its conversion rate rather than starting with a mixture of the enantiomers. The (*S,S*) CB-cyclam enantiomer was dissolved in toluene and then heated at 82 °C in a sealed container. Aliquots were taken at time intervals over 350 hours. Prior to heating, the initial sample of (*S, S*) CB-cyclam was analyzed by HPLC. The chromatogram obtained for this sample is shown in Figure 4.7. It is clear that there is already about 2.5 % of (*R,R*) CB-cyclam enantiomer in the sample.

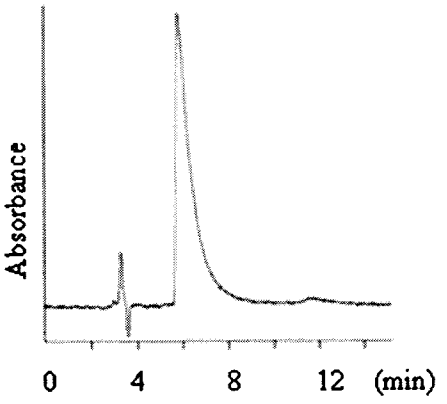


Figure 4.7. Chromatogram of (S,S) CB-cyclam prior to heating injected at a concentration of 0.10 mg/mL
Mobile Phase: 15/85/0.15 (v/v/wt%) ethanol/hexane/diethyl amine
Flow rate: 1.0 mL/min Column: Chiralpak AD (250 × 4.6 mm; 10μm))
Detection λ: 237 nm Temperature: 23 °C Injection: 10 μL

For each of the samples the amount of the enantiomer was determined based on the peak heights for its band in the chromatograms. The amount of the enantiomers was determined using Equation 4.1. The raw data for the peak heights are shown in Table 4.7.

$$\text{(S,S) enantiomer} = \frac{\text{Peak height (S,S) enantiomer}}{\text{Peak height (S,S) enantiomer} + \text{Peak height (R,R) enantiomer}}$$

(Equation 4.1)

		Peak height		
Time	**Peak**	**Injection 1**	**Injection 2**	**Injection 3**
0.00	Peak 1	171.2	174.3	147.3
0.00	Peak 2	4.5	4.2	4.4
66.67	Peak 1	168.6	166.4	165.2
66.67	Peak 2	10.2	9.7	10.5
154.08	Peak 1	180.9	181.1	178.4
154.08	Peak 2	22.5	23.9	21.8
243.93	Peak 1	160.3	165.2	167.9
243.93	Peak 2	29.4	29.4	28.8
349.95	Peak 1	191.4	195.6	193.4
349.95	Peak 2	50.3	48.2	46.3

Table 4.7. Raw data for the peak heights in mm

Enantiomeric excess (*ee*) was determined using the following equation (Equation 4.2):[10]

$$ee = \frac{|R - S|}{|R + S|} \quad \textbf{(Equation 4.2)}$$

The rate of racemization for the conversion of the pure enantiomer into a mixture of enantiomers was calculated using Equation 4.3, where k_{rac} is the racemization rate constant. [10] Equation 4.3 is then further simplified to give Equation 4.4.

$$\ln\left[\frac{1+\frac{[S]}{[R]}}{1-\frac{[S]}{[R]}}\right] = k_{rac}t \quad \textbf{(Equation 4.3)}$$

where
[S] = concentration of (S,S) CB-cyclam
[R] = concentration of (R,R) CB-cyclam
k_{rac} = rate constant for racemization
t = time

$$\ln ee = k_{rac}t \textbf{ (Equation 4.4)}$$

A plot of ln *ee* vs. time for the racemization of CB-cyclam (Figure 4.8) was constructed based on the chromatographic data obtained. The data fit to a linear model, which is consistent with first-order kinetics of racemization. The fit generated the equation $y = -3.31 \times 10^{-7}x - 0.0366$, with a slope of -3.31×10^{-7} which is rate constant (k_{rac} = -slope). Using the Eyring Equation (Equation 4.5), $\Delta G^{\ddagger}_{355.15}$ was calculated to be 31.44 ± 0.03 kcal/mol. Possible errors quoted in this $\Delta G^{\ddagger}_{355.15}$ are from the error in the slope, which is $0.156\times 10^{-7}s^{-1}$. In addition, for the measurement of the peak height there are only two significant figures therefore the $\Delta G^{\ddagger}_{355.15}$ measured by the chromatographic method is 31 kcal/mol by this method.

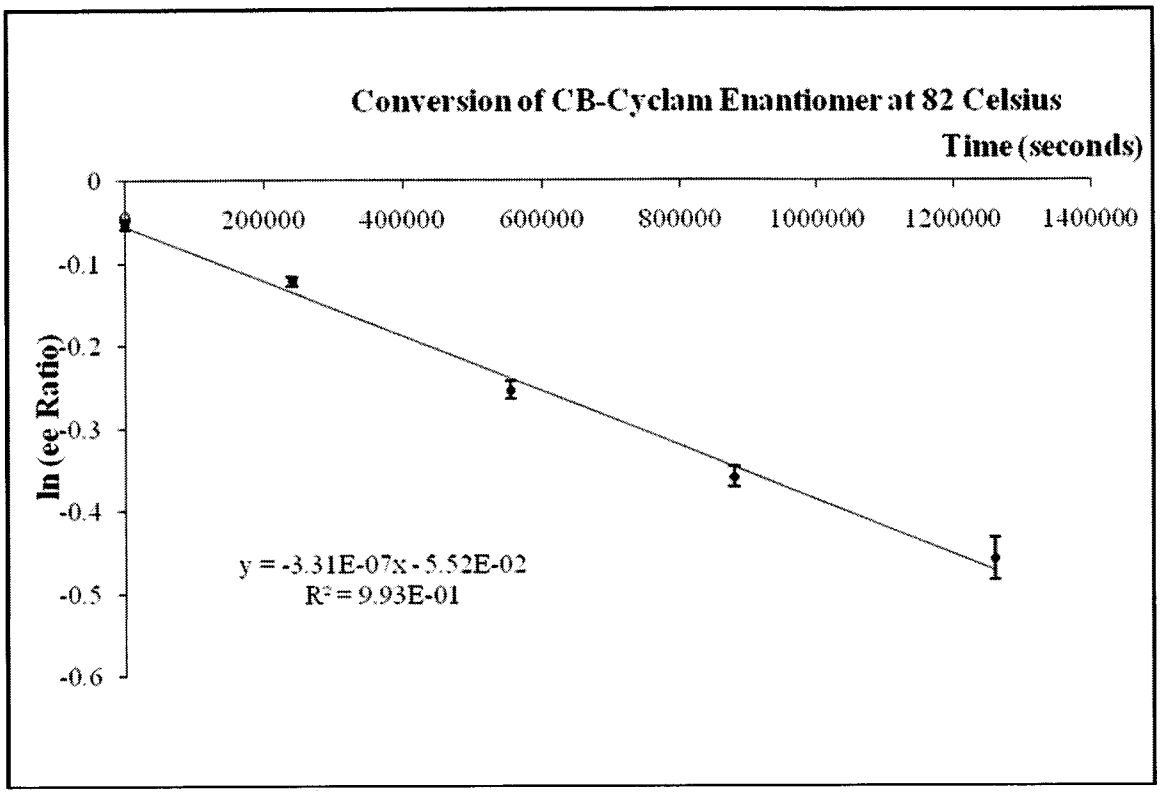


Figure 4.8. Plot of ln *ee* vs. time

$$k_{rac} = \left(\frac{\kappa k_b T}{h}\right) e^{\frac{-\Delta G^{\ddagger}}{RT}} \quad \textbf{(Equation 4.5)}$$

$$\Delta G^{\ddagger} = RT[\ln\left(\frac{T}{k_{rac}}\right) + \ln\left(\frac{\kappa k_b}{h}\right)] \quad \textbf{(Equation 4.6)}$$

$\Delta G^{\ddagger}$ is in cal/mol
T = absolute temperature in K
κ = transmission coefficient =1
k_b = Boltzmann's constant = 1.380658×10^{-16} erg·K^{-1}
h = Planck's constant = $6.62605755 \times 10^{-27}$ erg·s
R = Molar gas constant = 1.987215 cal·mol^{-1}·K^{-1}

The racemization barrier of CB-cyclam measured using NMR spectroscopy was found to be 31.42±0.07 and 31.29±0.10 kcal/mol on two separate runs at 82 °C.[10] The enantiomerization barrier for CB-cyclam using HPLC is within 5% of the value found by

NMR method. This demonstrates that HPLC may be used as an alternative method to NMR for evaluating the enantiomerization barrier of the cross-bridged polyamine enantiomers. HPLC provides a fast and less labor-intensive method for the determination of the enantiomerization barrier.

4.4 Conclusion

Conditions which resulted in the resolution of selected polyaza analytes on three different stationary phases (Chiralcel OD-R, Chiralpak AD and Chiralpak IB) were established. The chromatographic behavior of CB-cyclam, dibenzocyclam bisaminal, and dimethyl dibenzo-CB-cyclam for these stationary phases as a function of mobile phase concentrations, temperature, and additives were evaluated. The presence of additives such as diethylamine or diethanolamine significantly improved the resolution and peak shape for all analytes. The use of acidic additives such as ethanesulfonic acid is in general not desired as it results in significantly longer retention of the enantiomers. Only the enantiomers of dibenzocyclam bisaminal can be resolved without the addition of an additive. An increase in temperature would be desirable as it affords improved peak shapes and shorter analysis times. The enantiomers of dibenzocyclam bisaminal can be resolved on all stationary phases, while CB-cyclam enantiomers can only be baseline resolved on Chiralpak AD and dimethyl dibenzo-CB-cyclam can only be baseline resolved on Chiralcel OD-R. The maximum resolution for the CB-cyclam enantiomers was achieved using a 15/85/2 (v/v/wt %) ethanol/hexane/diethanolamine mobile phase on Chiralpak AD stationary phase. For the CB-cyclam and dibenzocyclam bisaminal enantiomers on the Chiralpak AD stationary phase the best separations were achieved at

ethanol ranges from 5 to 15 % by volume in hexane using 0.3 wt% diethylamine as an additive. On the Chiralcel OD-R stationary phase the enantioseparation was achieved at water ranges from 2 to 10% by volume in acetonitrile using 0.3 wt% diethyl amine as an additive. The resolution obtained is sufficient to evaluate the enantiomeric purity for any of the three cross-bridged tetraazamacrocycles. The speed of these separations would allow for an efficient scaleup and collection of individual enantiomers. The enantiomerization barrier for CB-cyclam was found to be 31 kcal/mol. Thus, in addition to NMR spectroscopy, chromatographic methods can be used to determine the enantiomerization barrier of cross-bridged tetraazamacrocycles.

CHAPTER V

CONCLUSION AND FUTURE STUDIES

The development of separation conditions for a wide range of copper complexes helped establish trends in their chromatographic behavior. The results showed that an increase in ring size of the ligands results in increased retention of the corresponding metal complexes under reversed phase conditions for different stationary phases including C_{18}, porous graphitic carbon and silica hydride stationary phases. In addition, the more hydrophobic the pendant arms of the ligands the stronger their related complexes' interactions on the C_{18}, silica hydride and porous graphitic carbon (PGC) stationary phases. The results obtained indicate that for the complexes studied the hydrophobicity of the ligand's pendant arm influences the retention to a greater degree than ring size under reversed phase conditions. Furthermore the results will assist in choosing chromatographic conditions in the future for copper(II) complexes with similar ligands to those studied.

The determination of physico chemical parameters for the copper (II) complexes is important because they are useful in predicting their *in vivo* behavior. A model study with Cu-cyclen and Cu-cyclam demonstrated that the chromatographic method is suitable for measurement of conditional formation constants through a competition reaction. The estimated log conditional formation constant for Cu-cyclen was 20.834

while the theoretical log conditional formation constant was 21.422, the two values are within 2.74% of each other. The slow kinetics of reaching equilibrium for Cu-CB-TE2A and cyclam made it impractical to use chromatographic methods for the measurement of the conditional formation constant of

Cu-CB-TE2A. The long time needed to reach equilibrium made it impractical to estimate the conditional formation constants of other ligands including Cu-CB-cyclam and Cu-CB-TE1A. Though the conditional formation constant for copper (II) complexes with cross-bridged ligands was not determined, acid inertness results suggest that they are better ligands at least kinetically than non cross-bridged macrocycles.

Other important physico chemical parameters include the pK_a and lipophilicity. The pK_as of the Cu-CB-TE2P complex were estimated to be around 4.0 - 4.1 for the first pK_a and around 6.2 - 6.3 for the second pK_a. The lipophilicity parameters for a series of methanephosphonate pendant-armed copper(II) complexes was determined and compared with those of carboxymethyl pendant armed complexes. The results showed that complexes with ligands of same ring size and comparable pendant arm size, the only difference being with one compound having a methanephosphonate group and on the other a carboxylate group, had similar lipophilicities. The k'_w for Cu-CB-TE2P was found to be 5.2 which is comparable to the published value for Cu-CB-TE2A k'_w of 5.9.[33]

An important aspect of this work was to develop HPLC methodology to assess the purity of copper(II) complexes. In addition, the development of chromatographic methods for producing chiral separations of selected cross-bridged tetraazamacrocyclic ligands allows for a fast and reliable method to determine enantiomeric purity. The use

of the developed chiral chromatographic methods to quickly produce enantiomerically pure material needs to be investigated further. Chiral chromatography could be an alternative to recrystallization for producing the desired enantiomer. The results showed that chromatographic methods can be used to determine the enantiomerization barrier for cross-bridged ligands. It is important to determine the enantiomerization barrier to establish the storage conditions and stability of an isolated pure enantiomer.

There are several research projects that could be initiated to advance the findings of the research presented in this thesis.

i) Conditional formation constants

A new approach could be taken to utilize HPLC for the determination of conditional formation constants for copper(II) complexes. The addition of a transfer ligand such as iminodiacetic acid can facilitate the transfer of the copper cation from one complex to the other so the system will achieve equilibrium faster.[67] The conditional formation constant for complexes of other metals including zinc, indium, and gallium with the cross-bridged tetraazamacrocyclic ligands might be evaluated using this method.

ii) Stationary phase evaluation

The silica hydride stationary phase needs to be evaluated further for the possible separation of the tetraazamacrocyclic ligands. This phase has shown increased retention for selected copper(II) complexes with methanephosphonate or carboxymethyl pendant armed ligands, including CB-TE2P and CB-TE2A. Due to retention mechanisms in addition to the hydrophobic interactions it is viable for the ligands to be retained and separated on this stationary phase. The development of chromatographic methods for the separation of these ligands would be useful for determining their purity prior to complexation with a metal ion. Furthermore the chromatographic methods can be used to

monitor reaction progress as the ligand peak is expected to disappear as the complex is formed. The lipophilicity of the ligands can be established after development of chromatographic conditions and correlated to the lipophilicity of their respective metal complexes.

Another stationary phase evaluated for the separation of selected copper(II) complexes was the PGC stationary phase. The PGC stationary phase was investigated particularly for the separation of charged copper(II) complexes. This study demonstrated that several charged complexes including Cu-cyclam, Cu-cyclen, Cu-CB-TEAMA, and Cu-CB-TE1A are retained on the PGC stationary phase. There are several charged complexes of indium (III) and gallium (III) with the CB-TE2A, CB-DO2A, CB-cyclam ligands including complexes that need to be investigated on this stationary phase. The development of chromatographic conditions for the indium and gallium complexes is important as it allows for a method to assess purity and follow reaction progress.

iii) pK_a determination and lipophilicity studies

In this study it was determined that Cu-CB-TE1A1P is pH active in the pH range of 5 to 9. Therefore it will be possible to measure the pK_a for Cu-CB-TE1A1P by monitoring its chromatographic behavior in the pH range of 5 to 9. The type and concentration of buffer needs to be investigated as to whether or not it plays a role in the determination of the pK_a. Buffers are organic in nature and they can affect the chromatographic retention of the analyte on the hydrophobic C_{18} stationary phase. The type of buffer needs to be investigated as it is possible to have interactions between the charged Cu-CB-TE1A1P and the buffer. In addition the role that the buffer plays when determining the pK_a should be investigated. A preliminary investigation of a series of closely related copper tetraazamacrocycles on other stationary phases such as a phenyl

based stationary phase might be useful. The Cogent C_{18} bidentate is a stationary phase that has well capped silanols. It has been shown that interaction with the underlying silanols can interfere with the calculation of the lipophilicity parameter. A comparison of the chromatographic behavior of the copper complexes on each stationary phase will also allow for the elucidation of the retention mechanism on the individual stationary phases.

iv) Separation of cross-bridged polyamine ligands and their metal complexes.

The development of chiral chromatographic conditions for other cross-bridged ligands including CB-homocyclen, CB-TE2A and CB-TE2P is important for evaluation of enantiopurity and the determination of the racemization barrier. In addition to exploring the enantioseparation on a chiral stationary phase the study should look at the possibility of achieving the resolution by addition of a chiral agent to the mobile phase. The addition of a chiral agent to the mobile phase will allow for the separation to be carried in an achiral stationary phase. The same stationary phase can be utilized for other separations while the chiral stationary phase is limited to chiral application. Furthermore, the development of enantioseparation conditions for the copper(II) complexes is important since they have potential use as cancer imaging agents. The enantiopurity of the copper(II) complexes can only be assessed through chromatographic conditions because NMR spectroscopy cannot be performed due to copper's paramagnetic properties.

The research projects mentioned above are only a few of the multiple research possibilities stemming from this research. Based on the results presented here the direct separation of charged metal ions might be investigated on the PGC stationary phase. This phase has demonstrated increased retention for charged complexes. The silica hydride stationary phase might be investigated for the separation of gallium and indium

complexes. The development of chromatographic conditions for gallium and indium complexes is important to assess complex purity and reaction completion.

Appendix I

Abbreviations list

Dibenzocyclam bisaminal : (13bα, 13cα)-5,6,7,12,13,13b,13c,14-Octahydro-4b,6a,11b,13a-tetraazadibenzo[b,def]chrysene

Dimethyl dibenzo-CB-cyclam: 5,12-Dimethyl-2,9-Dibenzo-1,5,8,12-Tetraazabicyclo[6.6.2]tetradeca-2,9-diene

Dibenzyl CB-cyclam: 4,11-dibenzyl-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane

Dimethyl CB-cyclam: 4,11-dimethyl-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane

DOTA-BA: 1,4,7,10-tetraaza-4,7,10-tris(carboxymethyl)-1-cyclododecylacetylbenzylamine

DOTA-MBA: 1,4,7,10-tetraaza-4,7,10-tris(carboxymethyl)-1-cyclododecylacetyl-R-(+)-alpha-methylbenzylamine

DTPA: diethylene triamine pentaacetic acid

DOTA: 1,4,7,10 – tetraazabicyclo-dodecane – N,N',N'',N''' –tetracetic acid

Cyclen: 1,4,7,10-tetraazacyclododecane

CB-cyclen: 1,4,7,10-tetraazabicyclo[5.5.2]tetradecane

Cyclam: 1,4,8,11-tetraazacyclotetradecane

CB-cyclam: 1,4,8,11-tetraazabicyclo[6.6.2]hexadecane

TETA: 1,4,8,11-tetraazacyclotetradecane - N,N',N'',N''' –tetracetic acid

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

CB-DO2LA: 4,10-bis(carboxyethyl)-1,4,7,10-tetraazabicyclo[5.5.2]tetradecane

CB-TE2LA: 4,11-bis(carboxyethyl)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane

CB-TE1A: 4-carboxymethyl-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane

CB-TEAMA: 4-carboxymethyl, 11-(2-amino2-oxoethyl) -1,4,8,11-tetraazabicyclo[6.6.2]hexadecane

CB-DO2P^{OEt}: 4,10-bis(methanephosphonic acid diethyl ester)-1,4,7,10-tetraazabicyclo[5.5.2]tetradecane

CB-TE2P^{OEt}: 4,11- bis(methanephosphonic acid diethyl ester)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane

CB-TE2P: 4,11- bis(methanephosphonic acid)-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane

CB-TE1A1P: 4- methanephosphonic acid, 11-carboxymethyl-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane

List of References

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