HPLC CHARACTERIZATION METHOD OF A CHEMOSENSING ENSEMBLE FOR HISTIDINE

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ABSTRACT

Chemosensing ensemble, a fertile area, is elucidating the nature and dynamics of small molecules, such as amino acids, binding to DNA. In particular, the design of site conformation-specific probes, selective cleaving agents for mapping and fingerprinting, and the potential in drug design drive this work. Traditionally, work has focused on small interactive binders with extended π system heterocycles having high imidazolate and sometimes carboxylate affinity. A major difficulty of this chemosensor design has been the lack of a clear understanding of the receptor-guest interactions that play a critical role in performance when these are characterized by HPLC method. Covalent systems provide means of attaching amino acids that bind tightly enough to the metal complex-receptors. In this work, the two Cu^{II} centers of the dimetallic complex of the bis-p-xylylBISDIEN was synthesized as a designed receptor for the recognition of histidine. This provides selective recognition of the ambidentate imidazole residue of histidine prepositioned at the right distance within BISDIEN ligand. The characterization method reported in this paper is simple in its preparatory and determinative stages. While significant fluorescence quenching observed from the molecule upon binding with two Cu^{II} to the imidazole residue of histidine caused a very low fluorescence intensity emission detected from HPLC characterization, a high specific of nonfluorescent products were observed by the optimized chromatographic gradient elution coupled with a UV-DAD detector measured at 338 nm and an Hypersil ODS column were utilized for analysis. It has been demonstrated that the peak areas were proportional to the concentrations of our receptor, [L·6HBr-Cu_2^{II}], after the optimum gradient conditions were performed. Linearity of the calibration curve was also very good (r^2 = 0.984). Minimum concentrations of histidine detection at 0.5 mM of reduced and nonreduced receptors were 1 μM and 0.5 μM, respectively with the specific retention time 1.93 min.

KEYWORDS: HPLC method, Chemosensing ensemble, Histidine, Transition metal complex, Imidazole group.

1. INTRODUCTION

Transition metal complexes (TMCs) with long-lived excited states are revolutionizing much of modern photochemistry and photophysics. In particular, copper and zinc-group metal complexes with guanidium subunits and/or diimine ligands have form the basis for several entirely new areas. Here we focus on system exhibiting fluorescence and the applications of these fluorescent properties to practical devices-in particular, molecular probes and sensors [1]. Sensors are molecules that respond to some property or analyte.We focus on fluorescence-based sensors employing TMCs. Widely used fluorescent detection methods are emission intensity, spectrum, excited state lifetimes and emission polarization. Basic sensing strategies for detecting and quantitating analytes include : 1. Specific excited state deactivation with a change in emission intensity. 2. Reaction of the ground or excited state species with the analyte to yield a species with different properties. 3. Variations on the previous cases where a nonemissive portion of the fluorescent species can react with the analyte and then after the fluorescent properties.

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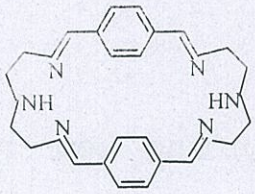
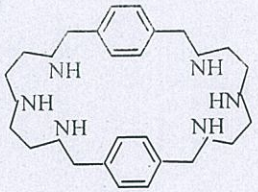
The problem of selective attachment to substrates for fluorescent molecular probes (or sensors) is a critical issue. One must design the necessary chemistry into the TMCs to allow attachment to the target. Since the ligands are generally organic, all the tools currently used to attach organic probes should be equally applicable to TMCs [2]. Covalent attachment as well as intercalation, ionic attraction, hydrophobic interactions, and guest-host interactions, provide means of attaching sensors to substrates. While such attachment is generally weaker than covalent systems, it does not preclude very successful utilization, as shown by widely used organic probes using these binding modes.The problem is creating metal complexes that bind tightly enough to the substrate, and this is a synthetic issue.

The development of fluorescent molecular sensors for some amino acids using metal ions, especially, the cations with biological interest such as Fe^{III}, Cu^{II} and Zn^{II}, has always been of particular interest [3-5]. Copper is the third in abundance (after Fe^{III} and Zn^{II}) among the essential heavy metals in the human body and plays an important role in various physiologic processes. The design and synthesis of chemosensors for amino acids, especially, for selective detection of histidine has become a very active area of research as a result of the demand for more sensitive and selective chemosensors for in vitro and in vivo purposes [4]. We describe now a novel type of 'chemosensing ensemble' for histidine using HPLC method. This chemosensor does not require the establishing of any covalent linking between the fluorophore and the receptor, which is in the form of TMCs, but utilizes the fluorophore and the receptor as such. Selective binding of histidine requires a receptor capable of interacting with the imidazole residue rather than with the carboxylate group, which is common to all the amino acids and which cannot, therefore, induce any selectivity. The extremely weak acid imidazole (pK_A = 14.5), in the presence of two Cu^{II} ions, prepositioned at the right distance within an appropriate ligand, deprotonates and bridges the two metal centers. As in this research, we select to use an imidazolate moiety bridges the two Cu^{II} centers of the dimetallic complexes of the bisdien macrocycle **L** and **L·6HBr**.

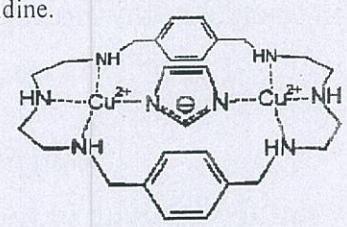


L



L•6HBr

In particular, each Cu^{II} centers in the ternary complex **[L-Cu_2^{II}-His]** becomes four-coordinate, according to a square geometry [3]. Thus, we decide to use the **[L-Cu_2^{II}]** and **[L·6HBr-Cu_2^{II}]** as a receptor for the recognition of histidine.



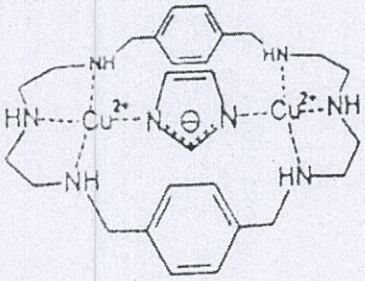
[L – Cu_2^{II} – His]

HPLC, one of the most powerful methods for amino acid analysis, usually require extensive preparatory work [6,7]. The optimal resolution can be achieved by correct selection of mobile phase, pH, organic modifier, and appropriate gradient profile. HPLC gradient elution of amino acids had been previously explained to be preferred because of the wide range of polarities of their derivatives and the expediency of time [6]. The type of elution used depends on the objective of the analysis. We considered that HPLC characterization method with suitable gradient conditions could provide a convenient framework for our receptor as chemosensing ensemble to recognize histidine.

2. MATERIALS AND METHODS

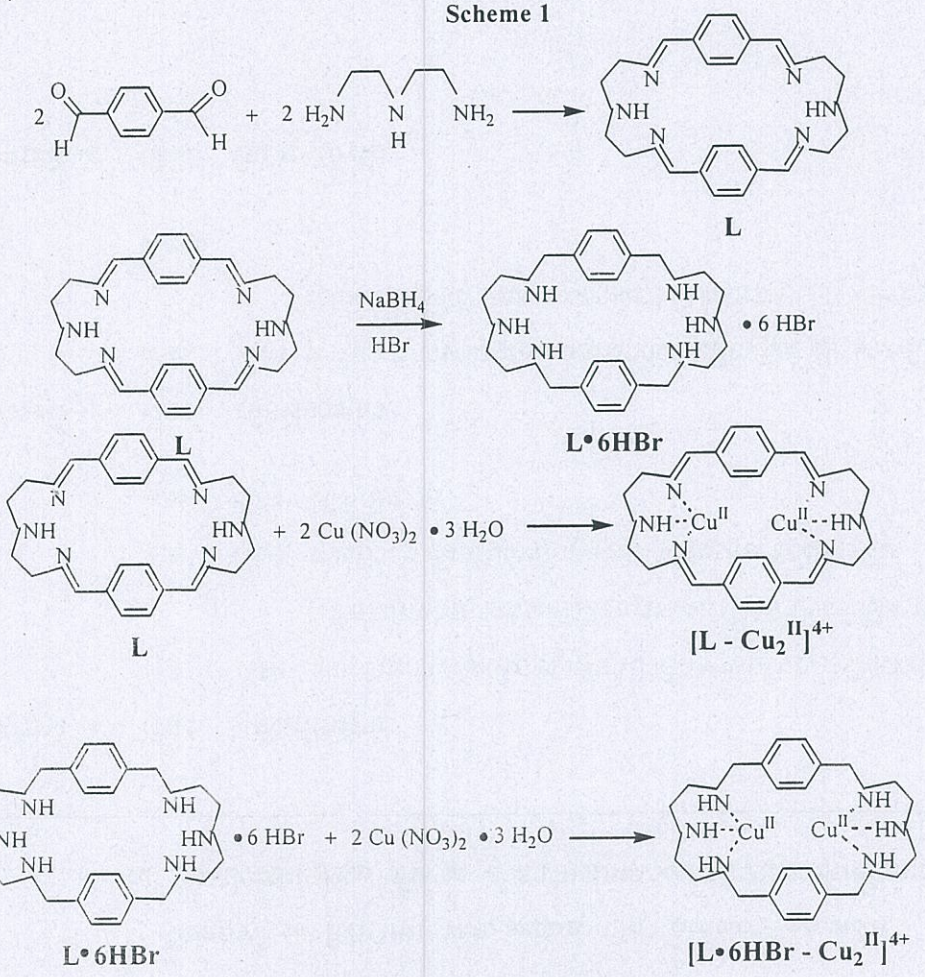
Reagents. Acetronitride and methanol (HPLC grade), dichloromethane, absolute ethanol, and 48% hydrogenbromide (AR grade) were purchased from Ajax Chemical (AUS). Benzene-1,4-dicarboxaldehyde, fluorescein and copper nitrate (AR grade) were purchased from Fluka.

Diethylenetriamine (AR grade) was bought from Sigma. Sodium acetate (HPLC grade) and sodium borohydride (AR grade) were purchased from APS Finechem.Tetrahydrofuran (HPLC grade) was purchased from Labscan Asia and lastly, histidine standard (AR grade) was bought from Hi Media Laboratory (India). All of reagents were used without further purification.



Syntheses. Macrocycles **L** and **L·6HBr** were prepared as previously described [8-10]. The complex $[L\text{-}Cu_2^{II}]$ $(NO_3)_4$ and $[L{\cdot}6HBr\text{-}Cu_2^{II}]$ $(NO_3)_4$ were prepared by dissolving two sets of 1.52 g. (6.28 mmol) of Cu $(NO_3)_2{\cdot}3H_2O$ in CH_3OH and adding each solution to a CH_3OH solution of 1.29g. (3.14 mmol) of **L** and **L-6·HBr,** respectively. The resulting solutions were heated to reflux, and after 30 min. the complex $[L\text{-}Cu_2^{II}]$ $(NO_3)_4$ and $[L{\cdot}6HBr\text{-}Cu_2^{II}]$ $(NO_3)_4$ which formed were collected by filtration, as bright blue powders **(scheme 1)**. Yield : 53.26% for $[L{\cdot}6HBr\text{-}Cu_2^{II}]$ $(NO_3)_4$. CHN analysis, by CHNS/O Analyzer (Perkin Elmer Series II, Model 2400), were found : %C 71.77, %H 8.59 and %N 20.18 for **L** and %C 31.28, %H 4.73 and %N 13.15 for **L·6HBr,** respectively. (calculate : C 36.69, H 4.87, N 17.68).

Scheme 1



Instrumentation. The UV-visible absorption measurements were recorded on Perkin Elmer (Lambda 35) spectrometer. ^1H and ^{13}C NMR spectra were obtained in CD_2Cl_2, CD_3OD and D_2O used

as purchased. Spectra were recorded on a Bruker AM-400 and Varian Gemini 2000 instruments. FTIR spectra were recorded on Bio-Rad FTS 175 spectrometer. Magnetic susceptibility measurements were performed by using Evan's method. R and R_0 values were reported with reference to $MnCl_2$ standard solution and were measured on Sherwood Scientific MKI version 1.40 Magnetic Susceptibility balance (MSB). χ_g , χ_M and μ_{eff} were calculated. A Varian Prostar high performance liquid chromatograph with 20 µL sample loop injector, cooled autosampler, temperature-controlled column compartment and Star Chromato-graphy Work Station V.6 Software recorder was used.

Histidine Standards. A stock solution was prepared by dissolving the equivalent of 32.2 µmol of histidine standard in 0.05 M NaOAc buffer. This stock solution was then used to mix with [L-Cu_2^{II}] or [L·6HBr-Cu_2^{II}] solution prepared as a working solution from which a calibration curve was prepared.

HPLC Characterization. This method is based on that of Schuster [7] with several modifi-cations. A Varian liquid chromatograph with gradient elution was used. The HPLC columns, a Phnomenex Spersorb 5 ODS 5 µm particle size, 4.6 mm x 15 cm and a Valco microsorb MV C_{18} , 4.6 mm x 15 cm (Varian, USA.) were used with a guard column. Gradient elution was generated using Prostar 230 solvent delivery system equipped with dual pumps and a Varian Prostar 400 injection valve containing a 20 µL sample loop. Star Chromatography Work Station V.6 was used for controlling the gradients and flow rate of the two degassed mobile phases. A Varian Prostar 363 programmable fluorescence detector fitted with a 5 µL flow cell was used with an excited monochromator setting in the range 330-524 nm and an emission cutoff filter of 418-540 nm. The other detector, A diode array detector, at wavelength 337 nm was also used for detection. The operating and chromatographic gradient conditions for HPLC analysis of chemosensing ensemble for histidine are given in **TABLE 1** and **TABLE 2**.

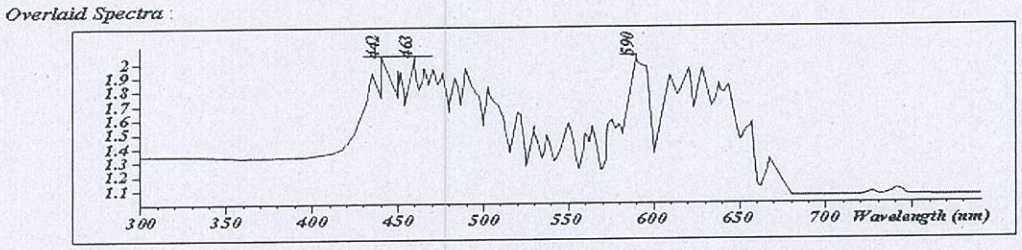
TABLE 1 : Operating Conditions for HPLC Analysis of Chemosensing Ensemble for Histidine.

Sample	Mobile phase A	Mobile phase B	Flow –rate (ml/min)	Column	Wavelength (nm)	Detector
1	0.05 M NaOAc (pH 5.9) : CH_3OH : THF (80 : 19 : 1)	0.05 M NaOAc (pH 5.9) : CH_3OH (1 : 4)	1.7	Phnomenex Spersorb5 ODS	λ_{ex} = 330 λ_{em} = 418	Fluorescence
2	0.05 M NaOAc (pH 4.6) : CH_3OH : THF (80 : 19 : 1)	0.05 M NaOAc (pH 4.6) : CH_3OH (1 : 4)	1.5	C_{18}	λ_{ex} = 330 λ_{em} = 418	Fluorescence
3	0.05 M NaOAc (pH 4.6) : CH_3OH : THF (80 : 19 : 1)	0.05 M NaOAc (pH 4.6) : CH_3OH (1 : 4)	1.5	Phnomenex Spersorb5 ODS	λ_{ex} = 424 λ_{em} = 487	Fluorescence
4	0.05 M NaOAc (pH 4.6) : CH_3OH : THF (80 : 19 : 1)	0.05 M NaOAc (pH 4.6) : CH_3OH (1 : 4)	1.5	Phnomenex Spersorb5 ODS	λ_{ex} = 489 λ_{em} = 513	Fluorescence
5	0.05 M NaOAc (pH 4.6) : CH_3OH : THF (80 : 19 : 1)	0.05 M NaOAc (pH 4.6) : CH_3OH (1 : 4)	1.5	Phnomenex Spersorb5 ODS	λ_{ex} = 524 λ_{em} = 540	Fluorescence
6	0.03 M NaOAc (pH 7.2) : 0.5% THF (1 : 1)	0.10 M NaOAc : ACN (1 : 4)	0.45	Phnomenex Spersorb5 ODS	λ = 338	UV – DAD
7	0.03 M NaOAc (pH 7.2) : 0.5% THF (1 : 1)	0.10 M NaOAc : ACN (1 : 4)	0.50	Phnomenex Spersorb5 ODS	λ = 338	UV – DAD
8	0.03 M NaOAc (pH 7.2) : 0.5% THF (1 : 1)	0.10 M NaOAc : ACN (1 : 4)	0.70	Phnomenex Spersorb5 ODS	λ = 338	UV – DAD

TABLE 2 : Chromatographic Gradient Conditions for HPLC Analysis of Chemosensing Ensemble for Histidine.

Time (min)	% A	% B	Flow – rate (ml/min)
0.00	80	20	0.45
1.00	83	17	0.70
2.00	83	17	0.70
3.00	83	17	0.70
4.00	83	17	0.70
5.00	83	17	0.45

3. RESULTS AND DISCUSSION



#	Name	Peaks (nm)	Abs (AU)
1		462.0	1.95980
1		442.0	1.95980
1		589.0	1.95980

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* * * End Spectrum/Peak Report * * *

Figure 1 : UV-visible spectrum of [**L·6HBr-Cu_2^{II}**].

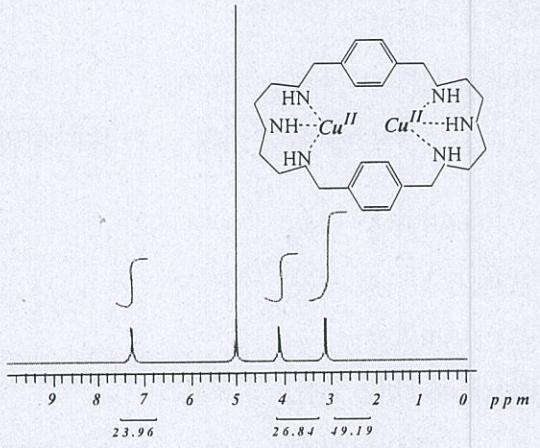


Figure 2 : Selected **^{1}H NMR** spectrum of [**L·6HBr-Cu_2^{II}**].

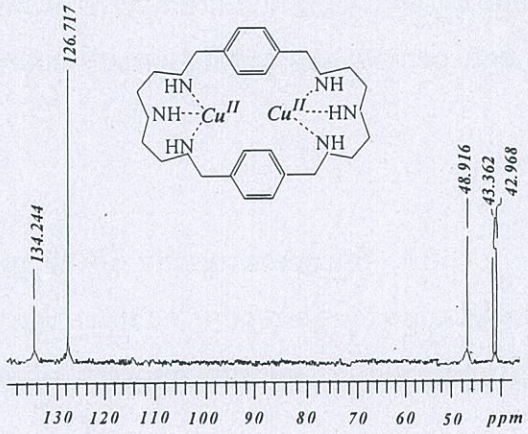


Figure 3 : Selected **^{13}C NMR** spectrum of [**L·6HBr-Cu_2^{II}**].

UV-visible Spectra. **Figure 1** shows the UV-visible spectrum of [L·6HBr-Cu_2^{II}] which exhibits 3 maximum wavelengths : 442, 463 and 589 nm that correspond to electronic spectra for the d-d transitions of Cu (II) complexes provide valuable information on the stereochemistry around the Cu (II) center. The electronic transitions have been shown to split into two bands with a splitting of ~ 700-1000 cm^{-1} and appear at about 21739-22523 cm^{-1} while the another band display at 17007 cm^{-1}. This seems to reflect a significant distorted planar three-coordinate Cu (II) centers.

^{1}H and ^{13}C NMR Spectra. **Figure 2** and **Figure 3** show the selected ^{1}H NMR in CD_2Cl_2 and ^{13}C NMR spectra of **[L·6HBr-Cu_2^{II}]** which exhibits well-resolved paramagnetically shifted resonances that span in the range 2.72-7.20 ppm for ^{1}H NMR and ~ 43.0-134.24 ppm for ^{13}C NMR as summarized in **TABLE 3**.

TABLE 3 : NMR Data for **[L·6HBr-Cu_2^{II}]**.

NMR spectrum	Chemical Shift (δ)
^{1}H (in CD_2Cl_2, 300 K)	2.72 (s, H, CH_2 of DIEN)
	3.75 (s, H, CH_2 of p-xylyl)
	4.60 (s, H, Ar-H)
	7.19 (s, H, Ar-H)
^{13}C (300 K)	42.968, 43.362 (CH_2 of DIEN)
	48.916 (CH_2 of p-xylyl)
	126.717, 134.244 (Aromatic C)

FTIR Spectra. Infrared spectroscopy has been widely used to establish the mode of bonding in complexes containing C-C, C-N, N-H and especially, M^{n+}_N groups. **Figure 4, 5** and **6** show the selected room-temperature FTIR spectra of **L**, **L·6HBr** and the complex of **[L·6HBr-Cu_2^{II}]**, respectively.

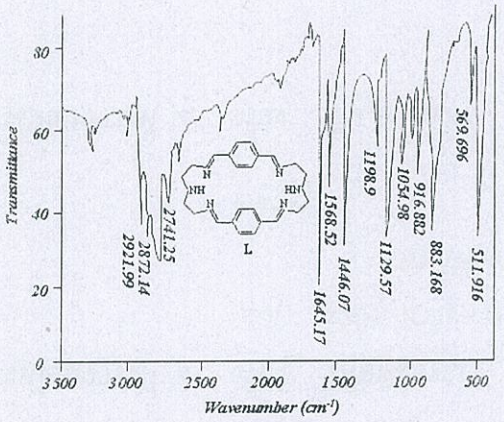


Figure 4 : IR-spectrum of **L** at 300 K.

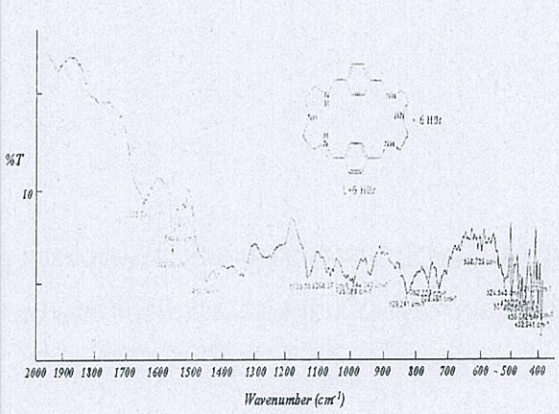


Figure 5 : IR-spectrum of **L·6HBr** at 300 K.

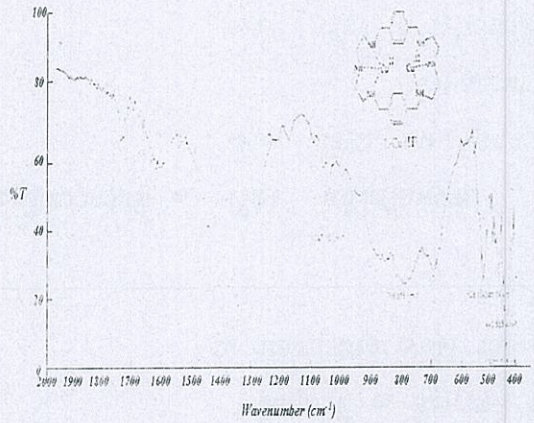


Figure 6 : IR-spectrum of **[L·6HBr-Cu_2^{II}]** at 300 K.

TABLE 4 : Average Magnetic Susceptibility Data.

Complex	C_{Bal} [a]	χ_g x 10^5 (cgs.)	χ_M x 10^3 (cgs.)	μ_{eff} (BM)	n
[L – Cu_2^{II}]	1.03	1.077	8.665	4.43	2
[L.6HBr – Cu_2^{II}]	1.03	0.694	5.454	3.60	2

[a] C_{Bal} are calculated from $C_{tube}/R-R_0$ at 298 K using standard $MnCl_2$ with C_{tube} = 1091, R_0 = -33 and R = 1022.

Paramagnetic Study. Magnetic susceptibility measurement has received something of a revival in popularity as a result of the search for new materials and the availability of the Evans balance from Sherwood Scientific. Measurements on the isolated solid compounds implicated **(TABLE 4)** the expected values of two unpaired electrons for two Cu (II) centers. The gram and molar magnetic susceptibilities (χ_g and χ_M) in cgs units were taken into account by use of equation (1) and (2) :

$$\chi_g\,(cgs) = \ell C_{Bal}\,(R\text{-}R_0) / 10^9 m \qquad \text{-----(1)}$$

$$\chi_M\,(cgs) = \chi_g \,.\, MW \qquad \text{-----(2)}$$

where ℓ = the sample length, R = the reading obtained for tube plus sample, R_0 = the empty tube reading and m = the sample mass. Some oxidations of Cu (II) complexes have been shown to produce radicals delocalized around an unsaturated macrocyclic BISDIEN rather than the true Cu (II) species with the unpaired electron associated with the metal center [12].

A more precise technique to study this problem may be found in ESR spectroscopy and electrospray technique under the conditions that were attempted. Unfortunately, for this work, we can not receive the further ESR and electrospray spectra to confirm our magnetic data. However, it is sharply clear that the qualitative interpretation of the present study is straightforward for one unpaired electron at each Cu (II) center. Any delocalized radical anion from macrocyclic ligand would be expected to give μ_{eff} higher than calculated values.

HPLC Results with UV-DAD detector. [L·6HBr-Cu_2^{II}] complex as a receptor for the recognition of histidine was characterized by HPLC study. Effects of the receptor concentrations to peak and at the retention time 1.933 min were report in **Figure 7** and corresponding to chromatogram in **Figure 8**.

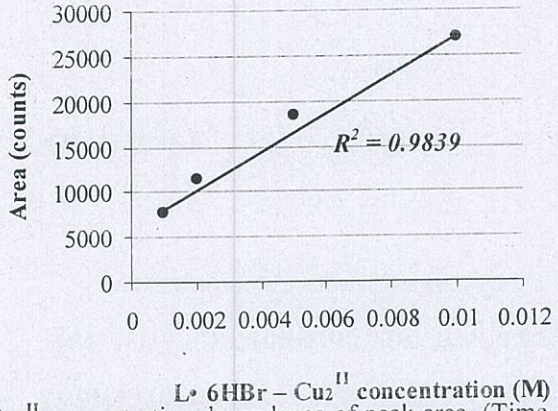
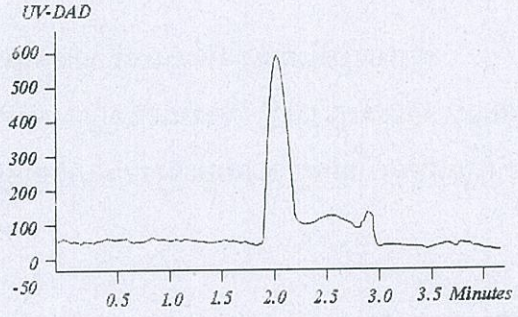


Figure 7 : L·6HBr-Cu_2^{II} concentration dependence of peak area. (Time 1.933 min, chromato-graphic gradient conditions as in **TABLE 1** and **2**).

Data File:	c:\star\data\sunee\7-2-48\com-1-7-2-2548
Channel:	1 = 338 nm RESULTS
Sample ID:	com-1
Operator (Inj):	van
Injection Date:	07/02/2005 13:09:38
Injection Method:	c:\star\install\histidine.mth
Run Time (min):	3.983
Workstation:	POWELL1
Instrument (Inj):	Varian Star #1
Operator (Calc):	van
Calc.Date:	07/02/2005 13:28:49
Times Calculated:	2
Calculation Method:	com-1-7-2-2548-1.mth
Instrument (Calc):	Varian Star #1
Run Mode:	Analysis
Peak Measurement:	Peak Area
Calculation Type:	External Std.
Calculation Level:	N/A
Verification Tolerance:	N/A



Peak No	Result	Peak Name	Ret. Time (min)	Time Offset (min)	Area (Counts)	Rel Ret Time	Sep. Code	Width ½ (sec)	Status Codes	Group
1	0.3315	Peak 1.250	1.933	-0.110	4161	0.00	BB	13.6		0
	0.3315	Totals		-0.110	4161					

Figure 8 : Chromatogram of L·6HBr-Cu_2^{II} receptor. Condition as in **TABLE 1**and **2**).

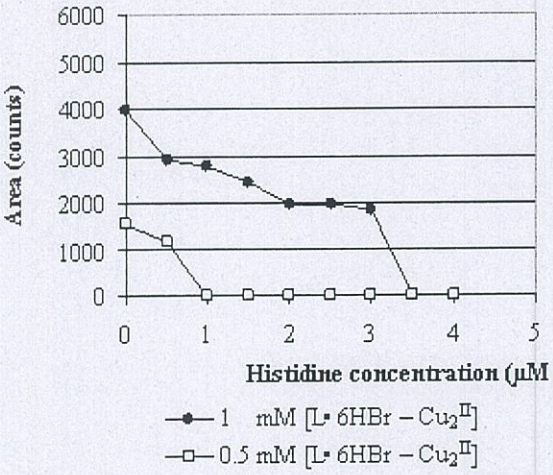


Figure 9 : Histidine concentration dependence of peak area using **[L·6HBr-Cu_2^{II}]**. (Time 1.92 min, chromatographic gradient conditions as in **TABLE 1** and **2**).

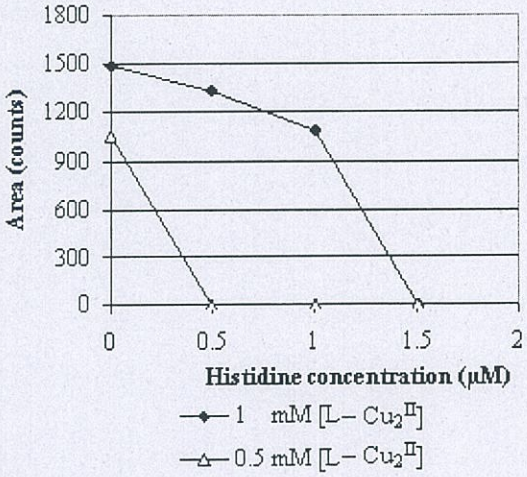
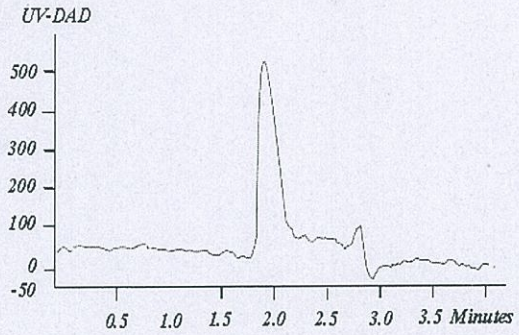


Figure 10 : Histidine concentration dependence of peak area. using **[L-Cu_2^{II}]**. (Time 1.92 min, chromatographic gradient conditions as in **TABLE 1** and **2**).

Data File:	c:\star\data\sunee\7-2-48\his-200-1-7-2-2548
Channel:	1 = 338 nm RESULTS
Sample ID:	his-200-1
Operator (Inj):	van
Injection Date:	07/02/2005 14:28:04
Injection Method:	c:\star\install\histidine.mth
Run Time (min):	3.982
Workstation:	POWELL1
Instrument (Inj):	Varian Star #1
Operator (Calc):	van
Calc.Date:	07/02/2005 14:32:08
Times Calculated:	1
Calculation Method:	c:\star\install\histidine.mth
Instrument (Calc):	Varian Star #1
Run Mode:	Analysis
Peak Measurement:	Peak Area
Calculation Type:	External Std.
Calculation Level:	N/A
Verification Tolerance:	N/A

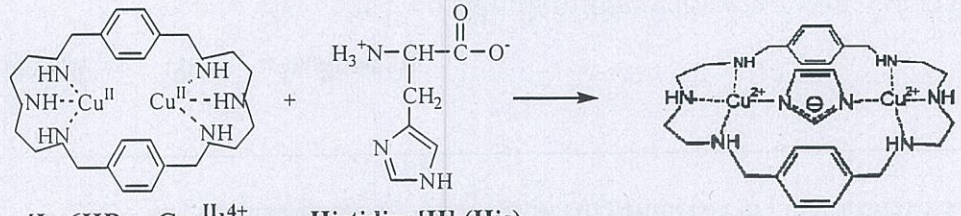


Peak No	Result	Peak Name	Ret. Time (min)	Time Offset (min)	Area (Counts)	Rel. Ret Time	Sep. Code	Width ½ (sec)	Status Codes	Group
1	0.0000		1.921	0.000	2787	0.00	BB	11.4		0
	0.0000	Totals		0.000	2787					

Figure 11 : Selected chromatogram of 1 mM **L·6HBr-Cu_2^{II}** receptor with histidine 10.0 nM

Our complex receptors are capable to interact with the imidazole residue of histidine rather than with the carboxylate group, which is common to all the amino acids. It is suggested that for the receptor/histidine, 1 : 1 adduct was formed as shown in **scheme 2**.

scheme 2



[L•6HBr - Cu_2^{II}]$^{4+}$ **Histidine[H] (His)**

The basicity of bisdien macrocycle **L** and **L·6HBr** is a function of the hybridization of the nitrogen atom.The more electronegative the nitrogen atom, the less readily it will shares its lone pair electrons and act as a base. The electronegativity of the nitrogen atom increases as the *s* character of the

hybridization increase, and hence its basicity decreases [13].In particular, discrimination would require that the basicity of **L** is distinctly lower than that of **L·6HBr**, then, any covalent linking between the two Cu^{II} center and **L** is weak. Additionally, it has been found that strained sp^2-ring system of **L** is much more reactive in reactions involving loss of positive Cu^{II}. Therefore, the stability and the chemosensor capacities of **[L-Cu_2^{II}]** are clearly less than of **[L·6HBr-Cu_2^{II}]**.

Effect of histidine concentration to peak area for selected various **[L·6HBr-Cu_2^{II}]** concentra-tions are shown in **Figure 9** with chromatogram shown in **Figure 11**. For nonreduced receptor **[L-Cu_2^{II}]**, HPLC results clearly show the less efficient receptor than reduced receptor. It can be suggested that each sp^2-N atom in **[L-Cu_2^{II}]** would reduce basicity of donor atom itself. Less binding of histidine by the nonreduced receptor was confirmed with HPLC data shown in **Figure 10**. It is clearly demonstrated that the more stability of the receptor structure, the more increasing in efficiency for acting as chemosensing ensemble. Since the amount of histidine detection depends directly on the receptor concentration and our stable nonreduced complexes are less than the stable reduced ones (at the same concentration), minimum concentrations of histidine detection at 0.5 mM of **[L-Cu_2^{II}]**, therefore, is slightly lower than that of **[L·6HBr-Cu_2^{II}]**.

HPLC-Fluorescence Detector Response. The mechanism of this chemosensing ensemble in recognition and sensing to histidine by the complex receptor of two Cu centers is shown and reported. We obtained very low fluorescence intensity and stability at the standard retention time of the receptor/histidine although their concentrations were varied in the wide range. Thus, it can be suggested that all adducts can completely quench the proximate fluorophore through either an electron or energy-transfer process. The nonfluorescent chemosensor was generated and can not be detected by fluorescence detector.

4. CONCLUSIONS

HPLC Characterization is one of the alternative methods to detect histidine from the designed chemosensing ensemble without multicomponent fluorescent systems. In the present study, the use of TMCs as a receptor containing two Cu^{II}, prepositioned at the correct distance within a hexamine macrocycle is selected to allow attachment to the target (histidine). Among of the numerous methods known to be fluorescent sensors,HPLC with the optimum chromatographic gradient conditions and selected UV-DAD detector was very satisfactory for histidine recognition study because nonfluorescent products were received. Sensitivity, however, depends on the equipment used, as well as the stereochemistry of the receptor itself. Use of a particular method for histidine analysis depends on availability of instrumentation and purpose of analysis. It has been demonstrated that careful choice of the method reported in this paper is simple in its preparatory and determinative stages for the achieve selectivity in histidine recognition and sensing.

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