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DETERMINATION OF COMPLEXING ABILITIES OF LIGANDS FOR METAL IONS BY FLOW INJECTION ANALYSIS AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

II*. COPPER(II) COMPLEXES OF AMINOPOLYCARBOXYLIC ACIDS

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SUMMARY

A combination of flow injection analysis (FIA) and high-performance liquid chromatography (HPLC) was employed for the spectrophotometric detection of aminopolycarboxylic acids (L) and the determination of their complexing abilities. The ligand L was injected into a carrier stream of the coloured methylthymol blue complex of copper(II) at pH 5.5, thus forming a complex CuL. By recording the absorbance at 595 nm, the complexing abilities of aminopolycarboxylate ions could be calculated from their FIA peak heights. The HPLC technique was also employed for on-line determination of the complexing abilities of individual ligands in multi-component samples.

INTRODUCTION

Aminopolycarboxylic acids, as well as inorganic polyphosphates, have been extensively used as sequestering agents in various industrial fields: foods, pharmaceuticals, fertilizers, detergents, water treatments, etc. Much attention has also been focused on the effect of these complexing ligands and related high-molecular-weight ligands on environmental waters and biological cycles.

It was thus important to develop automated analytical techniques for the characterization of such complexing ligands. A series of studies[1] was undertaken to design analytical methodologies based on flow injection analysis (FIA)[2–4] and high-performance liquid chromatography (HPLC)[5]. These techniques were expected to enable not only the rapid spectrophotometric determination of colourless ligands such as aminopolycarboxylic acids and polyphosphates, but also the determination of their complexing abilities.

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In the first paper of this series[1] we reported the principle of the substitution reaction method. A colourless ligand, L, was injected into a solution of a coloured methylthymol blue complex of magnesium, MgMTB, at pH 10. The variation in absorbance due to the formation of a colourless magnesium complex MgL was dependent on both the concentration and the complexing ability of L:

$$\mathrm{MgMTB} + \mathrm{L} \rightleftarrows \mathrm{MgL} + \mathrm{MTB} \quad (1)$$

Rapid determination of L was possible by the spectrophotometric measurement of negative FIA signals at a wavelength, 605 nm, where the molar absorptivity of MgMTB was greater than that of free methylthymol blue (MTB). The complexing abilities of various ligands for Mg^{2+} were also evaluated from their relative peak heights or relative sensitivities.

Copper(II) is one of the essential elements which play a vital rôle in biological metabolism[6]. Various aminopolycarboxylic acids have been used to characterize the interactions of Cu^{2+} with biological macromolecules. In this study, a copper(II) complex of MTB, CuMTB, was used as a coloured reagent for the spectrophotometric detection of aminopolycarboxylic acids at 595 nm and the estimation of their complexing abilities for Cu^{2+} at pH 5.5. An FIA system, with a stopped-flow device, was used for the sensitive and rapid detection of the individual ligands. A combination of FIA and HPLC was found to be useful for on-line characterization of the individual ligands in multicomponent samples.

EXPERIMENTAL

All organic chemicals (Dojindo Labs., Kumamoto, Japan) were used without further purification.

Methylthymol blue, *i.e.*, the sodium salt of 3,3'-bis[N,N-di(carboxymethyl)aminomethyl]thymolsulphonphthalein, $C_{37}H_{43}N_2O_{13}SNa$, was used as the chromogenic reagent. Abbreviations[7] for the organic ligands used as samples are as follows:

EDTA for ethylenediaminetetraacetic acid, $C_{10}H_{16}N_2O_8$; CyDTA for *trans*-1,2-cyclohexanediamine-N,N,N',N'-tetraacetic acid, $C_{14}H_{22}N_2O_8 \cdot H_2O$; DPTA-OH for 1,3-diaminopropan-2-ol-N,N,N',N'-tetraacetic acid, $C_{11}H_{18}N_2O_9$; EDTA-OH for N-hydroxyethylethylenediamine-N,N',N'-triacetic acid, $C_{10}H_{18}N_2O_7$; GEDTA for glycol ether diamine-N,N,N',N'-tetraacetic acid, $C_{14}H_{24}N_2O_{10}$; EDDA for ethylenediamine-N,N'-diacetic acid, $C_6H_{12}N_2O_4$; HIDA for hydroxyethyliminodiacetic acid, $C_6H_{11}NO_5$; IDA for iminodiacetic acid, $C_4H_7NO_4$; Me-EDTA for 1,2-diaminopropane-N,N,N',N'-tetraacetic acid, $C_{11}H_{18}N_2O_8$; DTPA for diethylenetriamine-N,N,N',N",N"-pentaacetic acid, $C_{14}H_{23}N_3O_{10}$; NTA for nitrilotriacetic acid, $C_6H_9NO_6$; and EDDP for ethylenediamine-N,N'-dipropionic acid, $C_8H_{16}N_2O_4 \cdot 2HCl$.

Orthophosphate (P_1), KH_2PO_4, diphosphate (P_2), $Na_4P_2O_7 \cdot 10H_2O$, and triphosphate (P_3), $Na_5P_3O_{10} \cdot 6H_2O$, were purified if necessary and confirmed as pure by HPLC[8].

The CuMTB reagent used for all FIA and HPLC experiments was prepared by dissolving copper(II) nitrate and MTB in 0.16 *M* sodium acetate–0.02 *M* acetic acid buffer , pH 5.5 and comprised $7.0 \cdot 10^{-5}$ *M* copper(II) and $1.0 \cdot 10^{-4}$ *M* MTB.

The CuMTB reagent for the measurement of the absorption spectrum was an equimolar ($5 \cdot 10^{-5}$ *M*) mixture of copper(II) and MTB in the same buffer.

Eluents 1 and 2 for gradient elution were prepared by dissolving tetramethylammonium chloride in 0.08 *M* sodium acetate–0.001 *M* acetic acid buffer, pH 5.5 to yield 0.05 *M* $(CH_3)_4NCl$ and 0.20 *M* $(CH_3)_4NCl$, respectively.

The FIA system consisted of a reciprocating pump with two channels (Seishin PSU-3.2W), a spectrophotometer (Jasco, UVIDEC 100W) with a flow cell (8 μl), a loop-valve sample injector (Seishin VMU-6, 100 μl) and a switching valve for the stopped-flow (Kyowa KMU-4V2). The HPLC system (Jasco TRIROTAR) was equipped with an anion-exchange separation column (50 cm × 2.6 mm I.D., TSK-GEL, IEX220SA; alternative designation, TSK-GEL, SAX; 10 μm).

RESULTS AND DISCUSSION

The detection principle is based on a substitution reaction in which a colourless liagnd, L, to be determined reacts with a coloured CuMTB complex to form a colourless CuL complex:

$$\mathrm{CuMTB} + \mathrm{L} \rightleftharpoons \mathrm{CuL} + \mathrm{MTB} \quad (2)$$

As shown in Fig. 1, the molar absorptivity of the CuMTB complex, ε_{CuMTB}, is different from that of free MTB, ε_{MTB}, in the visible region. The absorbance of the reaction system 2 is expected to decrease with increasing concentration of L, if the spectrophotometric measurement is carried out at 595 nm where the value of $\varepsilon_{CuMTB} - \varepsilon_{MTB}$ is positive and as high as *ca.* $1 \cdot 10^4 M^{-1}$ cm^{-1}. A plot of the negative variances in absorbance *versus* sample concentrations can be used as a calibration graph for the determination of L.

It is also expected from eqn. 2 that the greater the stability constant of CuL, the higher will be the sensitivity of detection or the relative peak height. In other words, the order of complexing abilities of various ligands can easily be determined by measuring their relative FIA peak heights. The relative peak height of a given ligand L, H_L, is defined on the basis of the sensitivity of EDTA

$$H_L = \frac{h_L}{C_L} \cdot \frac{C_{EDTA}}{h_{EDTA}} \quad (3)$$

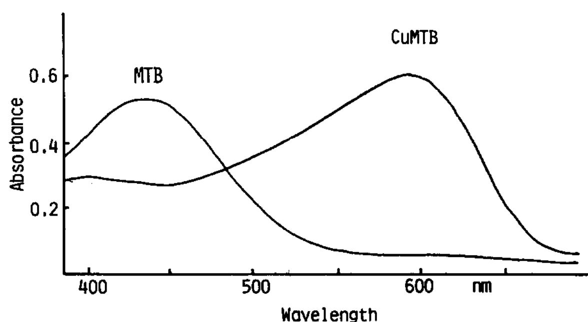

Fig. 1. Absorption spectra of MTB and CuMTB at pH 5.5. Concentration of each is $5 \cdot 10^{-5}$ *M*.

where h_L is the peak height at any concentration of L, C_L. Thus, $H_L = 1.0$ for EDTA, as $C_L = C_{EDTA}$ and $h_L = h_{EDTA}$. EDTA was selected as a standard ligand because the CuEDTA complex[7,9,10] is well characterized as a 1:1 complex with stability constant of $10^{18.80}\ M^{-1}$.

Both MTB and its copper(II) complex may be highly protonated at pH 5.5[7,9,10]. Therefore, CuMTB in eqn. 2 and Fig. 1 does not refer to the unprotonated CuMTB complex whose stability constant is *ca.* 10^{14}. The conditional stability constant of the CuMTB complex, K_{CuMTB}, at pH 5.5 is calculated to be *ca.* 10^7. This value need not be known exactly, because all the discussion is based on calibration methods using standard ligands under the given conditions[1]. It is also noted that all the aminopolycarboxylate ions used as samples may be protonated to some extent at pH 5.5. Therefore, this work concerns "conditional" complexing abilities of ligands at pH 5.5.

Some aminopolycarboxylate complexes of copper(II) are not strictly transparent in the visible region. However, CuL in eqn. 2 may be considered to be practically colourless because its absorption at 595 nm is neglibible compared with those of CuMTB and MTB.

A coupled system of FIA and HPLC is shown in Fig. 2. During the FIA experiments the HPLC system was disconnected from the FIA system at point M. A sample solution of L (100 μl) was injected via a loop-valve injector (S) into a carrier stream of water and mixed at point M with a stream of the coloured CuMTB complex. The flow-rates of the water and CuMTB streams were controlled by a reciprocating pump with two channels (plungers P_b and P_c) at 1.0 and 0.5 ml/min. In a reaction coil (RC) immersed in a water-bath (50°C) the sample reacted with the CuMTB reagent to form the CuL complex, resulting in a variation of absorbance. A negative peak[1] was recorded when the absorbance was monitored at 595 nm by employing a spectrophotometric detector (D) with a flow cell (8 μl). It should be noted that the input and output terminals of the detector were reversed so that negative peaks could be recorded as positive peaks, as in Figs. 3–5.

A T-joint with a section of closed tubing, T_d, was located after each pumping channel as a mini-air-damper. Any piece of hard PTFE tubing and flexible peristaltic

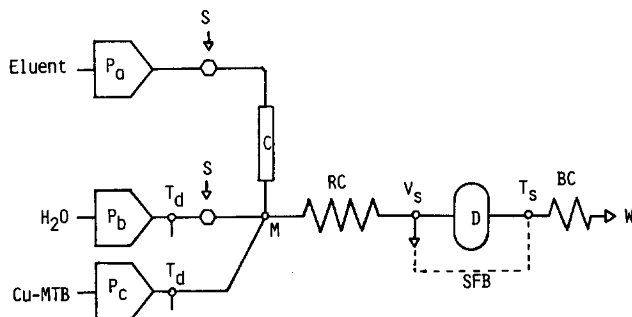

Fig. 2. A combination of FIA and HPLC. P_a, Pumping system for HPLC with a gradient device; P_b and P_c, reciprocating pumps for FIA; S, loop-valve sample injector; T_d, T-joint with a flexible air-damper tubing (*ca.* 20 cm × 0.6 mm I.D., Technicon 116-0536-6); M, mixing joint (three-way or four-way); C, separation column; RC, reaction coil (500 cm × 0.5 mm I.D., PTFE); BC, back-pressure coil (200 cm × 0.25 mm I.D., PTFE); D, detector; V_s, switching valve; T_s, T. joint; SFB, stopped-flow bypass (50 cm × 0.5 mm I.D., PTFE); W, waste.

tubing was satisfactory for damping the pulse of the reciprocating pump. The flexible tubing used in this work plays an additional rôle as a "fuse" for limiting the pressure increase (*ca.* 10 kg/cm^2) due to unexpected clogging of the analytical line and for preventing breakdown of the reaction coil and flow cell and the leakage of corrosive reagents.

A switching valve for stopped-flow experiments, V_s, was located just before the detector in order to observe the variation in absorbance of the sample zone. When the sample zone reached the flow cell the valve was switched to direct the carrier stream through a bypass (SFB) to waste (W) and to stop the sample zone in the flow cell. The extent of the reaction was determined by recording the variation in absorbance for a suitable period. The valve was then switched on-line through the detector to waste. The stopped-flow bypass was very effective in minimizing fluctuations of the sample zone in the detector.

An example of a calibration profile for EDTA is shown in Fig. 3. The baseline corresponds to the absorbance of the coloured CuMTB reagent at 595 nm. Each sample was injected in triplicate at 1.5-min intervals to record inverted negative peaks. In routine work with injections of 60 samples per hour, the relative standard deviation was 0.5%.

The last broad signal in Fig. 3 is that obtained at 50°C by stopping the sample zone, corresponding to the peak maximum, in the detector to observe the time dependence of the absorbance or the further progress of the reaction. The absorbance was expected to vary with time if the substitution reaction had not been completed[11]. The appearance of the stopped-flow signal as a plateau leads to the conclusion that the substitution reaction has almost reached equilibrium at 50°C. At 30°C, on the other hand, the stopped-flow signals of most ligands continued to increase gradually, suggesting further progress of the substitution reactions in the detector. All experiments described hereafter were carried out at 50°C.

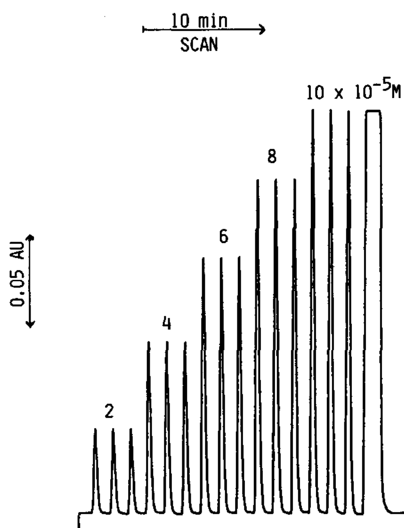

Fig. 3. FIA calibration signals for EDTA in the CuMTB system. Each sample volume is 100 μl. Each sample is injected in triplicate, except for the fourth injection of $10 \cdot 10^{-5}$ *M* for the stopped-flow recording.

FIA profiles obtained with the CuMTB system are shown in Fig. 4 for twelve aminopolycarboxylic acids and three inorganic phosphates. The stopped-flow signals show that the substitution reactions of the aminopolycarboxylic acids, except GEDTA, are complete at 50°C. Each sample has a different sensitivity or peak height. Aminopolycarboxylic acids could be sensitively detected at a low concentration, $1 \cdot 10^{-4}$ M, whereas inorganic phosphates were undetectable at the same concentration. H_L values can be calculated according to eqn. 3, although there is some uncertainty in the C_L values of aminopolycarboxylic acids calculated on the basis of weighed amounts and formula weights. Of the aminopolycarboxylic acids in Fig. 4, the lowest H_L value found is 0.06 for IDA. H_L values for inorganic phosphates were determined at higher sample concentrations and found to be less than 0.005. As for the magnesium complexes employed previously[1], the H_L values increased with increasing conditional stability constant of the CuL complexes[7,9,10], K'_{CuL}, and tended to level off in the region where $K'_{CuL} \gg K'_{CuMTB}$, *e.g.*, for EDTA, EDTA-OH, Me-EDTA, CyDTA and DTPA with $H_L = 1$. The order of H_L values or complexing abilities of the ligands is: phosphates < IDA < HIDA < NTA < EDDA < DPTA-OH <

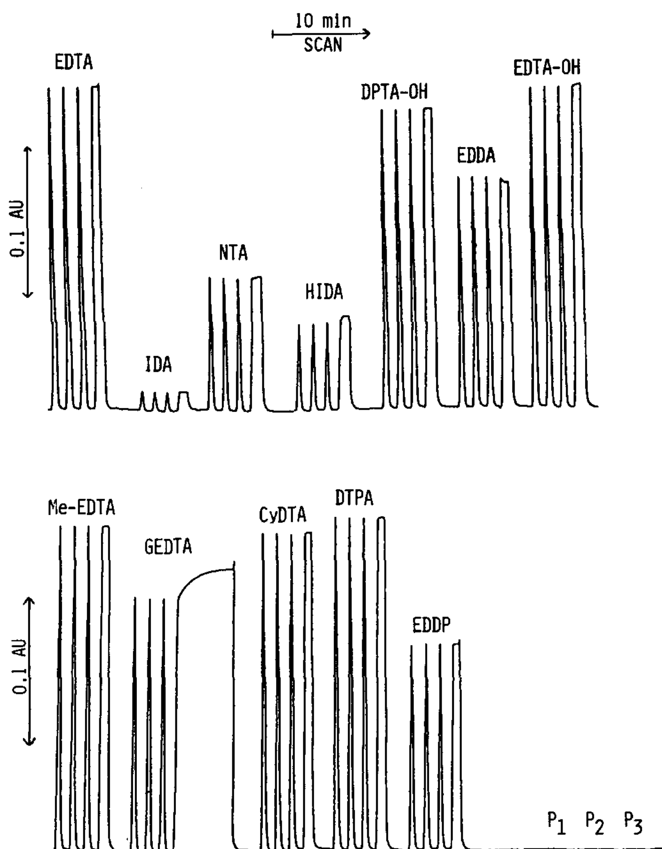

Fig. 4. FIA signals for twelve aminopolycarboxylic acids and three inorganic phosphates in the CuMTB system. Samples: each 100 μl and $1 \cdot 10^{-4}$ M, except for IDA ($1 \cdot 10^{-3}$ M). Each sample was injected in quadruplicate to record three FIA signals and a broad stopped-flow signal.

EDTA. It is of interest that the order of H_L values in the CuMTB system at pH 5.5 is in contrast with that in the MgMTB system at pH 10: IDA < EDDA < HIDA < P_2 < DPTA-OH < NTA < P_3 < EDTA[1].

As is seen in Figs. 3 and 4, the FIA method is very satisfactory for the determination of the complexing abilities of individual ligands, but does not allow differential analysis of multicomponent samples. Prior separation is needed for this purpose. The pumping channel P_b in Fig. 2 was disconnected and the HPLC system was activated to permit both separation and detection by post-column reaction[5,12–14] of aminopolycarboxylic acids. Fig. 5 shows HPLC profiles for mixtures of three aminopolycarboxylic acids (a) and of six aminopolycarboxylic acids (b). A gradient technique (Jasco GP-A30, Convex 1 mode, 64 min) was employed at the flow-rate of 1.0 ml/min with eluents 0.05 M $(CH_3)_4NCl$ and 0.20 M $(CH_3)_4NCl$ at pH 5.5. Similar results could be obtained by using potassium chloride solutions instead of tetramethylammonium chloride solutions.

Peak areas were measured (Shimadzu CHROMATOPAC, C-R1A) to determine the complexing ability of each ligand. The results are reasonably consistent with the complexing abilities obtained by FIA for individual ligands. A drawback associated with gradient elution is that the ionic strength of the stream in the detector cannot be kept constant, which may affect the sensitivities of detection. An isocratic elution is recommended for the exact quantitation of relative complexing abilities, if the problem of separation is not significant. If analytical separation is of major concern, the variation in ionic strength becomes insignificant, because calibration curves must be constructed in any case for individual chromatographic peaks.

All the experimental results described above are based on the measurement of substitution reactions between CuMTB and ligands at pH 5.5. Similar results can also be obtained at physiological pH 7.2 (Tris–HCl buffer) using the same CuMTB reagent.

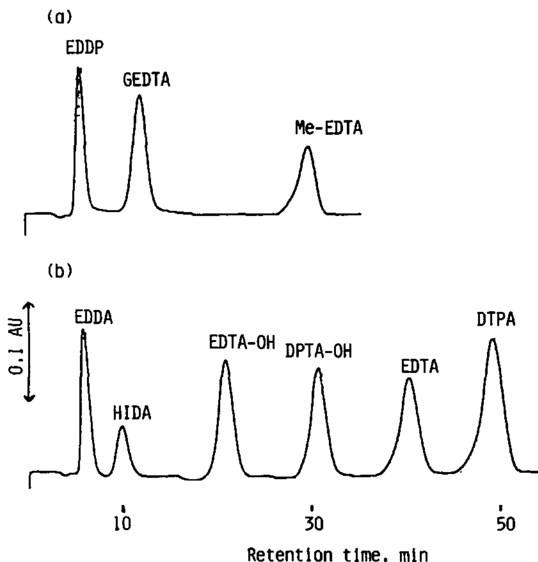

Fig. 5. HPLC profiles for a mixture of three aminopolycarboxylic acids (a) and of six aminopolycarboxylic acids (b). Samples: volume, each 0.3 ml; concentration, each $1 \cdot 10^{-4}$ M, except for DTPA ($2 \cdot 10^{-4}$ M).

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