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Article

Synthesis and Characterization of EDTA Complexes Useful for Trace Elements Supplementation

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A sintese de dois complexos de EDTA heterobimetalicos, $MgCu(EDTA).6H_2O$ e $ZnCu(EDTA).6H_2O$, foi descrita. Ambos foram caracterizados por espectroscopia vibracional (infravermelho e Raman) e eletrônica (reflectância). Alguns testes de dissolução em HCl 0.1 mol L^{-1} e em suco gastrico artificial foram também efetuados. Os resultados indicam o uso potencial desses complexos de cobre como suplemento medicinal humano e veterinário e ao simultâneo reforço dos níveis de Mg(II) e Zn(II).

The synthesis of two heterobimetallic EDTA complexes, $MgCu(EDTA).6H_2O$ and $ZnCu(EDTA).6H_2O$, is described. They were characterized by means of vibrational (infrared and Raman) and electronic (reflectance) spectroscopy. Several dissolution tests in 0.1 N HCl and simulated gastric juice were also performed. The results support the potential usefulness of these complexes for copper supplementation in human and veterinary medicine and to the simultaneous reinforcement of Mg(II) and Zn(II) levels.

Keywords: EDTA-complexes, IR spectra, Raman spectra, electronic spectra, dissolution assays, copper supplementation

Introduction

Supplementation of essential trace or microtrace elements is an area of increasing interest in the field of human and veterinary pharmacology. In particular, copper supplementation is a problem of wide, strong economic and clinical impact due to the fact that a series of well-known metabolic disorders and diseases found in both humans and animals are clearly related to copper deficiencies.[1-4]

After iron and zinc, copper is the third most abundant transition element in the human body [2,5,6] and it plays an important series of biological functions as it is involved in electron transport (for example, plastocyanin, azurin); in oxidase systems like amine oxidase, ascorbate-oxidase, galactose oxidase and lysyl oxidase; in oxygenases like tyrosinases, in the transport of oxygen (hemocyanins); in the dismutation of the superoxide radical (superoxide dismutase) and even in iron metabolism (ceruloplasmin).[2,3,5,6]

There are a number of disorders related to deficiencies or to elevate copper levels in the organism. Two hereditary diseases concerning copper metabolism are particularly well-known: Wilson's disease and Menkes' syndrome. Both of them occur with a probability of about 1 case per 10^5 births. Wilson's disease involves a pathological accumulation of copper in the liver and brain, and becomes lethal if left untreated.[2,5,7,8] Different chelating agents have been proposed to remove selectively the excess of accumulated copper, D-penicillamine being the most effective and widely used.[2,5]

In contrast to Wilson's disease, Menkes' syndrome is caused by a hereditary dysfunction of the intracellular copper transport and, thus, insufficient copper storage.[1,2,5] A characteristic symptom of this disease is the occurrence of "kinky" hair, illustrating clearly the involvement of copper-containing proteins in the biosynthesis and stabilization of structural and connective tissues. This disorder must be treated by continuous supplementation of the element. If supplementation is interrupted, a rapid and eventually lethal degradation occurs in the central nervous system.[5]

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Other genetic defects causing changes in the amino acid sequence of the important antioxidant enzyme Cu/Zn superoxide dismutase[2,5,6] are responsible for the familiar form of amyotrophic lateral sclerosis (ALS), a progressive and irreversible neurodegenerative disease of the motor system.[5] It has also been demonstrated that the copper status of humans and animals affects strongly the levels of neuropeptides.[1-3,5]

In the case of animals, manifestation of copper deficiency includes anemia, diarrhea, bone-disorders, reproductive failure, achromotrochia (loss of hair pigment) and keratinizacion failure in hair, fur and wool.[3,4] One of the best known environmentally caused disorders of copper metabolism in animals, especially in ruminants, is the molybdenum/copper antagonism.[2-5,9] It appears to result from a complexation of Cu(I) by $MoO_{4-n}S_n^{2-}$ species as formed under reducing conditions in the stomach of the ruminants. Thiomolybdates are readily soluble in water and are excellent chelating agents for metal centers in low oxidation states,[10] favouring in this way the copper excretion, generating a secondary copper deficiency.

Different copper compounds have been used for copper supplementation or for the treatment of different disorders and diseases. In the case of Menkes' disease the Cu(II) complex of L-histidine seems particularly effective.[1,5,11] The treatment with Cu(I) salts in the presence of sebacic acid have also considered as very promising[12,13] and even stoichiometric copper(II) sebacate[14,15] appears as potentially useful. Besides, different simple Cu(II) salts and complexes have been shown interesting anti-inflammatory, antiulcer, anticonvulsant and antitumoral activity.[1,2,16]

Different simple or complex compounds have also been used in veterinary pharmacology for the supplementation of this element. These included copper sulfate solutions, the Cu(II) complexes of glycine or methionine and some salts of ethylenediaminetetraacetic acid (EDTA), such as CuCaEDTA or $Na_2CuEDTA$.[4,9,17-20] Another often used system is copper oxide, supported in gelatin capsules, or in the form of soluble glass boluses containing other micronutrients (for example Co and Se).[17,20]

As part of a research project devoted to the characterization of copper complexes with recognized pharmacological activity, in the last years we have investigated in detail some general physicochemical properties of Cu(II) complexes of simple amino acids[21-25] and small peptides.[26-28] We also initiated some fieldwork, using a number of the investigated amino acid complexes for the supplementation of copper to ruminant's.[29]

As a continuation of these studies, we have now investigated two EDTA complexes, containing simultaneously copper(II) and zinc or magnesium.

EDTA (ethylenediaminetetraacetic acid) is one of the best known and most widely used chelating agents in both analytical chemistry[30] and medicine.[2,8] It is a potentially hexadentate chelating ligand, as each of the nitrogen atoms has a free electron pair and the molecule possesses four acidic hydrogens ($pK_1 = 2.0$, $pK_2 = 2.67$, $pK_3 = 6.16$, $pK_4 = 10.26$).[8] The $(EDTA)^{4-}$ anion is capable of complexing almost every metal cation in the Periodic Table and the six coordinating groups are able to occupy four, five or six coordination sites around a central metal cation. The degree of EDTA-metal complex formation normally depends upon the stability of the particular metal-complex and the pH-value of the environment.

For the three metal cations involved in the present study, the logarithms of the respective stability constants (β) defined as:

$$\beta = [M(EDTA)^{2-}]/[M^{2+}][(EDTA)^{4-}]$$

are respectively 8.69 (for Mg(II)), 16.50 (for Zn(II)) and 18.80 (for Cu(II)),[30] indicating the peculiar stability of the Cu(II) complex. This means that in the Cu(II)/Zn(II) and Cu(II)/Mg(II) systems, the copper(II) cation may be preferentially chelated by EDTA, whereas the other two metals probably remain as countercations, out of the coordination sphere. In fact, this behaviour has been proved by the structural analysis of a series of heterobimetallic EDTA complexes of the type $M(H_2O)_4[M'(EDTA)].2H_2O$ [31] that crystallizes in the same structure as $Zn_2(EDTA).6H_2O$ [32] and $Co_2(EDTA).6H_2O$,[33] space group $P2_1n$ and $Z = 4$. Two different coordination sites are present in these compounds and both are roughly octahedral. In the "hydrated" site, the M cation is coordinated to four water molecules and two *cis*-oxygen atoms belonging to two bridging carboxylate groups of side-chelated centers. In the "chelated" site, the M' cation is bonded to four oxygens (one from each carboxylate group) and the two nitrogens from the hexadentate EDTA ligand. Therefore, the lattice can be described as being constituted by *zig-zag* strings of alternating "hydrated" and "chelated" complex ions linked through carboxylate bridging groups.

Experimental

Syntheses

$MgCu[EDTA].6H_2O$. A mass of 2.9 g (10 mmol) of ethylenediaminetetraacetic acid were dissolved in 200 mL of distilled water, mixed with 0.80 g (10 mmol) of $Cu(OH)_2$ and heated after dissolution of the hydroxide. To this solution 0.87 g (slightly more than 10 mmol) of $MgCO_3$

were added in small portions, under vigorous stirring and heating. After half an hour, the remnant carbonate is filtered off and the solution concentrated over a water bath up to the crystallization of the product. The clear-blue crystals were separated and dried in air between filter papers.[34]

ZnCu[EDTA].6H_2O. This compound was prepared in a similar way as above, replacing $MgCO_3$ by $ZnCO_3$. After separation of the excess of carbonate and concentration of the solution to about 50 mL, the clear-blue crystals were precipitated by addition of cold methanol.[34]

Spectroscopic measurements

The infrared spectra were recorded with a Perkin Elmer 580 B spectrophotometer in the spectral range between 4000 and 200 cm^{-1}, using the KBr pellet technique. Raman spectra were measured, on the powdered samples of the complexes in a capillary tube, with a Jasco TRS-600/SZ-P instrument using the 514.5 nm line of an Ar^+ laser for excitation.

Electronic (reflectance) spectra were recorded with a Shimadzu UV-300 spectrophotometer using MgO as a standard.

Pharmacological assays

Dissolution tests were performed following the recommendations and procedures of the *U. S. Pharmacopoeia* (XXII. Edition).[35] The simulated gastric juice was obtained using NaCl/pepsine/HCl mixtures (final pH-value = 1.4).[35]

Results and Discussion

Synthesis

The preparation is very easy and the mixed-metal complexes are obtained with high yields and in a very pure form, as confirmed by the performed chemical analyses [for the Cu/Mg complex: Found: C, 25,00; H, 4.90; N, 5.85; Cu, 13.25. Calc. for $C_{10}H_{12}O_8N_2CuMg.6H_2O$: C, 24.80; H, 4.96; N, 5.79; Cu, 13.13%. For the Cu/Zn complex: Found: C, 23.05; H, 4.45; N, 5.40; Cu, 12.20. Calc. for $C_{10}H_{12}O_8N_2CuZn.6H_2O$: C, 22.86; H, 4.57; N, 5.33; Cu, 12.10%.].

The described synthetic strategy can also be applied for the preparation of other similar species.[31,34]

Vibrational spectra

The infrared and Raman spectra of the two investigated complexes are, as expected, very similar. The IR spectrum of the Cu/Mg complex is shown in Figure 1 and the corresponding Raman spectrum in Figure 2. The band positions measured for both complexes, together with the proposed assignment for the most characteristic vibrations are presented in Table 1. These assignments are mainly based on some general references [36-38] as well as on some specific spectroscopic studies of EDTA complexes [31,39-41] and similar Cu(II) chelates of amino acids.[23-25] Some aspects of these assignments are briefly commented below.

In the higher frequency region (not shown in the Figures) a very broad and strong IR-band, originated in

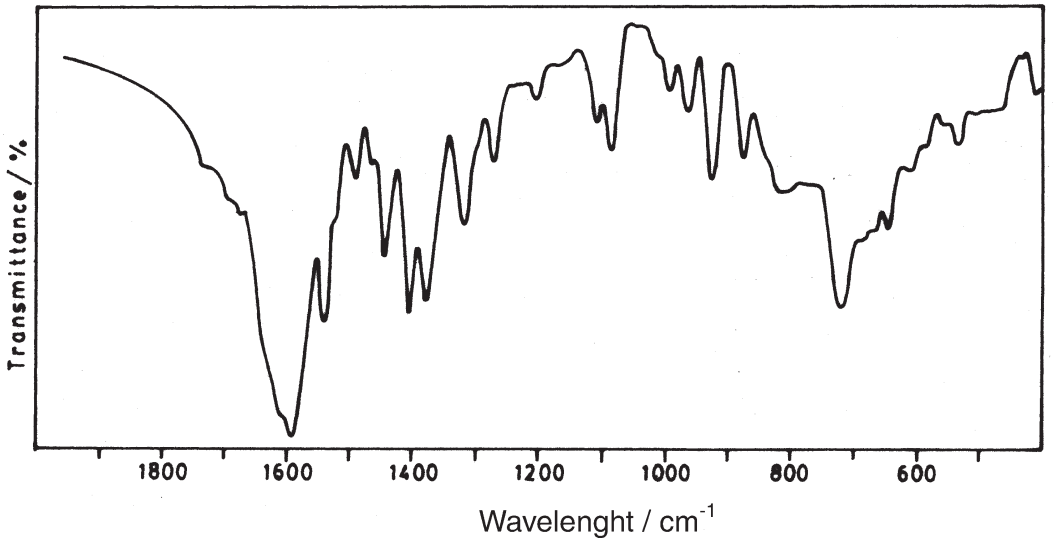

Figure 1. MgCu[EDTA].6H_2O complex: IR spectrum between 2000 and 400 cm^{-1}.

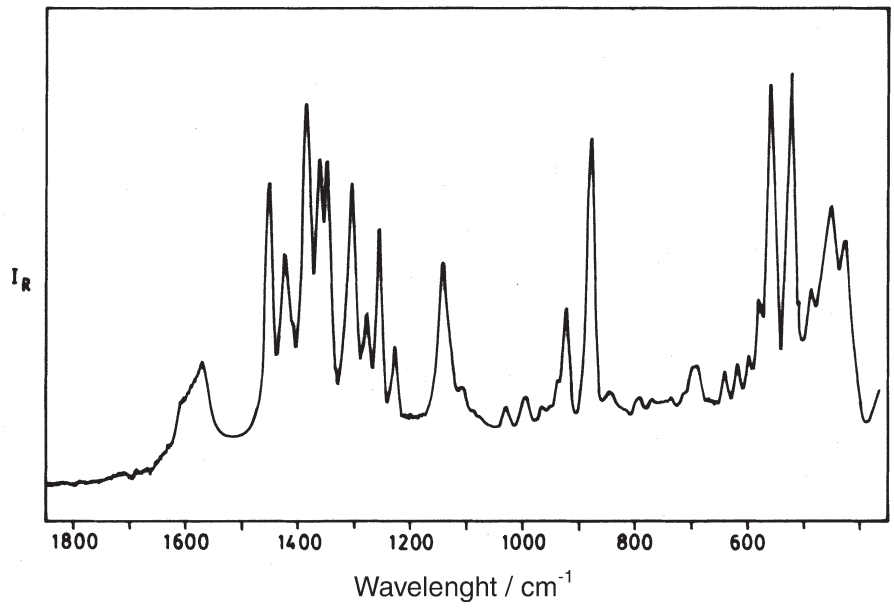

Figure 2. MgCu[EDTA].6H_2O complex: Raman spectrum between 2000 and 400 cm^{-1}.

Table 1. Vibrational spectra of MgCu(EDTA).6H_2O and ZnCu(EDTA).6H_2O in the spectral range between 1800 and 400 cm^{-1}

MgCu(EDTA).6H_2O		ZnCu(EDTA).6H_2O		Assignment
IR	Raman	IR	Raman	
1731sh,1692sh		1702 sh, 1683w		
1665 w		1665 sh		
1612 sh		1626 sh		
1592 vs	1595 w	1605 vs	1595 m	$\nu_{as}(COO^-)$ md
1575 sh	1570 m	1575 sh	1565 m	$\nu_{as}(COO^-)$ br
1541m,1524sh				
1491 m				
1446 s	1449 vs	1435 sh	1435 m	$\delta(CH_2)$
1407 s	1420 s	1401 s	1416 s	$\nu_s(COO^-)$ br
1378 s	1382 vs	1385 s	1382 vs	$\nu_s(COO^-)$ md
1360 sh	1360vs,1347vs		1341 m	$\delta(CH_2)$
1318 s	1300 vs	1319 s	1305 s	$\delta(CH_2)+ \nu(C\text{-}CO_2)$
1272 m	1274 m	1268 w	1270 sh	$\delta(CH_2)$
	1254 s		1248 w	
1205 w	1224 m	1227 w	1234 m	
1166sh	1140 s	1195 w	1138 s	ν(CNC)
1108m,1086m	1106w,1085vw	1107m,1080m	1060w,1045w	ν(CN)
1015vw,995w	1033 w,996w	1000vw,970w	985 w	
	968w,947sh			
927 s	927 m	927 m	934 m	ν(CC)
876 m	875 vs	867 w	876 vs	ν(CC)
812 m	827 w	812 w	810 w	
721 s	720 vw	733 m	761 w	$\rho(CH_2)$
690 vw	692 w	667 w	704 m	
646 w	645 w	635 w	615 vw	$\rho(H_2O)$ (?)
613 w	621 w	600 sh		
593 vw	604 w			
562 vw	570 vs	577 w	572 vs	see text
538 w	538 vs	525 vw	538 vs	see text
	498 w			
462 w	461 s	467 w	470 s	ν(Cu-N)
412vw, 392w	438 m	391 w	395 w	

Band intensities: vs: very strong; s: strong; m: medium; w: weak; vw: very weak; sh: shoulder.

the O-H stretchings of the six H_2O molecules, is observed for both complexes. It is centered at about 3425 cm^{-1} in the Mg/Cu complex and at 3435 cm^{-1} in the Zn/Cu complex. On the lower-frequency side of this broad band some features, at about 2960 and 2920 cm^{-1}, related to the stretching vibrations of the CH_2-groups were observed.

The HOH bending mode could not be identified with certainty, it surely contributes to the observed broadening of the central and most intense IR band around 1600 cm^{-1} as well as to the corresponding Raman band in the same spectral region. One of the vibrational modes of coordinated water was also tentatively assigned in Table 1.

As derived from the structural characteristics discussed above, two different types of carboxylate groups are present in the "chelated" site of these compounds, as two of them participated in bridge bonding between the two metal centers whereas the other two form only monodentate bonds with the Cu(II) centers. Therefore, one expects two groups of carboxylate stretching vibrations. In Table 1 they have been identified as md (for the monodentate groups) and br (for the bridging groups). The $\nu_{as}(COO^-)$ vibration of the second group is not easy to identify, as it only appears as a relatively weak shoulder on the lower frequency side of the main band, related to this same mode of the monodentated carboxylates. On the contrary, in the Raman spectra, both bands can be clearly differentiated. The corresponding $\nu_s(COO^-)$ vibrations are more cleanly separated in both spectra.

The mentioned carboxylate stretching vibrations satisfactorily fulfill some well-known criteria regarding its spectral position and the energy differences between the antisymmetric and symmetric vibration.[38] This energy difference ($\Delta\nu$) for the monodentate coordinating groups is 214 cm^{-1} in the Cu/Mg complex and 220 cm^{-1} the Cu/Zn complex. As expected, these $\Delta\nu$-values are clearly higher than those of the bridging carboxylate groups (168 cm^{-1} for Mg/Cu and 174 cm^{-1} for Cu/Zn).

In assigning deformational modes of the CH_2 groups we have not differentiated between different kinds of possible motions. Only the characteristic rocking (ρ) mode was separately identified. Some of these modes, as well as other skeletal modes are probably coupled together and can not be considered as "pure" vibrations.

Some relatively strong Raman lines in the spectral range below 600 cm^{-1} could not be confidently assigned. They are probably motions related to the CuN_2O_4-skeleton of the "chelated" site.

Regarding the metal-to-ligand vibrations, only one of the Cu-N stretching modes could be tentatively assigned. It lies in the same region in which this vibration is usually found in Cu(II) complexes of amino acids,[23-25] ammonia and ethylendiamine.[38] The Cu-O motions involving the carboxylate oxygen atoms, are expected to lie at around 300 cm^{-1}, whereas those related to the M-O vibrations involving the water molecules surely lie between 300 and 400 cm^{-1}.[38]

Electronic spectra

The electronic (reflectance) spectra of both complexes are practically identical, presenting a very strong and broad band centered at about 750-770 nm (13.33-12.98 kK). This band is characteristic of the $^2E_g \rightarrow {}^2T_{2g}$ "d-d" transition of the Cu(II) cation in an octahedral environment.[42,43] The broadness of this band is also usually observed in simple octahedral copper (II) complexes, as a consequence of the Jahn-Teller effect, expected to be very important in the case of a d^9 electron configuration.[42,43]

Dissolution assays

They were performed in simulated gastric juice and in 0.1 $mol.L^{-1}$ HCl in order to verify the solubility and the speed of dissolution of both compounds. To supply the lower-limit copper dose to a normal human adult (about 2 mg/day), 15.2 mg of the Cu/Mg complex and 16.5 mg of the Cu/Zn complex must be administered. The assays were performed with 20 mg of each of both compounds.

This dose of each compound was introduced into 250 mL of the simulated gastric juice and slowly stirred. After 2 minutes a totally clear solution was observed for both salts. These assays were duplicated, with the same results. This test confirms the dissolution of the compounds and the release of magnesium and zinc into the gastric juice. Judging by the color of the obtained dissolutions copper probably remains in chelated form as EDTA complex.

The same sample quantities as above were introduced into 50 mL of 0.1 $mol.L^{-1}$ HCl, maintained at 37 °C and stirred at a constant speed of 60 rpm. The dissolution times, obtained from two independent experiments for each compound, were similar to those obtained above, *i.e.*, less than 2 minutes for the dissolution of both compounds. This test accepted by the *U.S.P.* as an assay for dissolution of powders allows a maximum dissolution time of 30 minutes for one dose.[35]

We have also repeated these assays with higher doses (50-60 mg) to simulate basic animal requirements (see below). In these cases dissolution times were also very low, lying in the order of 3 minutes.

The commented results confirm a rapid release of the divalent metal cations and show that both compounds are potentially useful for copper supplementation. They could be used as a part of solid mixtures of micronutrients, in the form of aqueous solutions, or as an injectable solution.

Table 2. Minimum copper requirements for humans and animals (**a**), quantities of the two complexes to be administered to cover these minimum copper levels (**b**) and concentration of the simultaneously administered levels of Mg(II) and Zn(II) (**c**)

	a	**b**	**b**	**c**
	Cu(II)	Mg/Cu-complex	Zn/Cu-complex	Mg(II)/Zn(II)
humans	2 mg/d	15.2 mg	16.5 mg	0.76 / 2.05 mg
animals	7 mg/kg	53.3 mg	57.8 mg	2.68 / 7.20 mg

The investigated compounds may also be useful to supplement small quantities of Mg(II) and Zn(II) with each copper administered dose. For adult humans, the requirements of these two elements are in the order of about 300 mg/day (Mg(II)) [4,44] and 12-15 mg/day (Zn(II)).[4] For animals the doses range between 10-15 mg/kg (Mg(II)) [4,44] and 20-40 mg/kg (Zn(II)).[4]

In Table 2 the available quantities of each of the two metals are shown, related to the supplemented doses of copper. As it can be seen, the supplemented doses for both metals lie far away from the minimum requirements. Notwithstanding, one or other compound may be selected to ameliorate, simultaneously with the copper supplementation, zinc or magnesium levels, as needed in particular cases.

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