

Metal Complexes of Anhydrotetracycline. 3: An Absorption and Circular Dichroism Study of the Ni(II), Cu(II) and Zn(II) Complexes in Aqueous Solution

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Received: March 28, 1995; August 14, 1995

A anidrotetraciclina (AHTC) é o principal produto tóxico de degradação do antibiótico tetraciclina. A complexação da AHTC aos íons Ni(II), Cu(II) e Zn(II) em solução aquosa a pH 7 foi estudada por espectroscopia de absorção e de dicroísmo circular. Sugerimos a formação da espécie MH_2L_2 com Ni(II) e de uma espécie MHL tanto para o Cu(II) quanto para o Zn(II), pela coordenação da AHTC aos íons metálicos através das posições O11 e O12 do sistema de anéis BCD. Como se pode inferir da análise dos espectros de dicroísmo circular, nos complexos de Ni(II) e Zn(II) a AHTC adota provavelmente a conformação do zwitterion, LH_2 , na qual a proximidade entre os oxigênios O1, O11 e O12 favorece a coordenação do ligante de modo tridentado, enquanto no complexo de Cu(II) o ligante coordena-se provavelmente de modo bidentado, adotando uma conformação diferente da de LH_2 . Foram calculadas as constantes de estabilidade de todas as espécies.

Anhydrotetracycline (AHTC) is the major toxic decomposition product of the antibiotic tetracycline. The complexation of AHTC to Ni(II), Cu(II) and Zn(II) in aqueous solution at pH 7 was studied using absorption and circular dichroism (CD) spectroscopy. We suggest the formation of a MH_2L_2 species with Ni(II), and of a MHL species with either Cu(II) and Zn(II), by coordination of AHTC through the O11 and O12 positions in the system of the BCD rings. As may be inferred from the CD spectra, in the Ni(II) and Zn(II) complexes AHTC probably adopts the same conformation as the zwitterion LH_2 , in which the juxtaposition of O1, O11 and O12 allows the ligand to coordinate tridentately, whereas in the Cu(II) complex the ligand is probably bidentate, adopting a conformation different from that of LH_2 . The stability constants of all species were calculated.

Keywords: *anhydrotetracycline, metal complexes, conformations*

Introduction

Anhydrotetracycline, hereafter called AHTC (1) is one of the principal toxic degradation products of the antibiotic tetracycline (TC, 2). AHTC is responsible for several adverse side effects of the drug, such as the reversible Fanconi type syndrome and cutaneous phototoxicity upon ingestion of TC beyond its limit of validity^{1,2}.

Removal of a water molecule from the C5a-C6 positions of TC by treatment with warm mineral acid gives the anhydro derivative³. The crystal structure of AHTC hydrobromide reveals that the aromatization of ring C produces significant changes in the A and B rings relative to the corresponding ones in the parent compound, although the overall conformations of both compounds are similar⁴. The AHTC molecule is more planar in the C-D ring region than TC. A slightly different conformation of the A ring of

AHTC was also demonstrated, the nitrogen of the C4 position being closer to the C12a oxygen than to the C3 oxygen in the solid. The loss of activity of the anhydro compound is thought to be due to these structural differences⁴.

Like its parent compound, AHTC contains several potential binding sites for metal ions. In addition to the phenolate oxygens at C10 and C11, and the carbonyl one at C12 in the system for the BCD rings, the molecule presents different coordination positions on ring A, namely the amide and the dimethylamine groups at C2 and C4, respectively, and the enolic function at C3.

It was shown that the free forms of TC occur at insignificant levels in blood plasma during treatment, calcium and magnesium complexes being predominant in the fraction of the drug not bound to proteins⁵. It was also demonstrated that essential trace metal ions like zinc and copper are present in concentrations in the blood plasma too low to significantly influence the bioavailability of the drug⁶. In contrast, the interactions of the antibiotic with zinc were shown to be important in the gastrointestinal tract. In fact, it was observed that the metal plays an antagonistic role on the gastrointestinal absorption of the drug⁷. On the other hand, copper tetracycline interactions have been recently implicated in the degradation of DNA through free radical production in an intracellular process⁶.

On the basis of these results similar studies of the chelating properties of AHTC should be of considerable interest. Depending on the concentration of AHTC resulting from the ingestion of degraded TC, the influence of the metal complexes of the anhydro derivative on the toxic side effects, as well as on the lowering of the TC activity, should be appraised.

In previous reports^{8,9} we started a systematic investigation of the chelating ability of AHTC. The Cu(II) and Ni(II) complexes were prepared in the solid state⁸. Pursuing this, we performed a study of the interactions of AHTC with Mg(II), Al(III) and Fe(III) in aqueous solution, by absorption and circular dichroism (CD) measurements⁹. The present work deals with the interactions of AHTC with Ni(II), Cu(II) and Zn(II) in aqueous solution at pH 7, followed by absorption and CD spectroscopy.

Experimental

Apparatus

A Diode Array Hewlett Packard 8451 A spectrometer equipped with a Masterline 2095 thermostat at 25 °C and a Jobin Yvon Mark V dichrograph were used for UV and visible absorption and CD measurements, respectively. The pH measurements were obtained on a Metrohm 633 titroprocessor provided with a Metrohm EA 147 combined glass electrode.

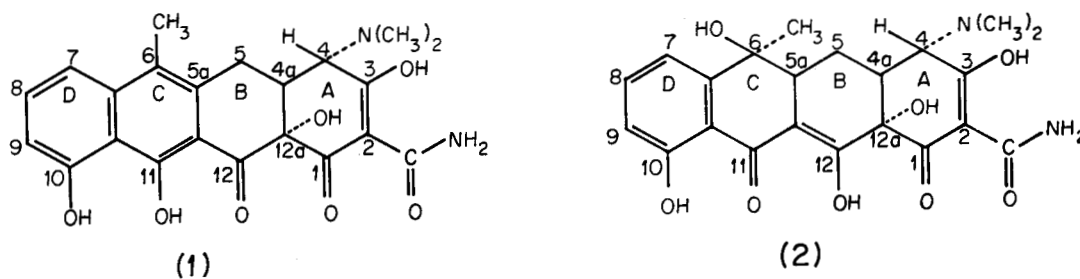
Chemicals

AHTC hydrochloride was obtained by the method described by L.J. Stoel *et al.*³. All other chemicals were of analytical quality.

Solutions and titration procedures

The interactions of AHTC with Cu(II), Ni(II), and Zn(II) ions in aqueous solution were followed using absorption and circular dichroism (CD) spectroscopy in the UV-visible range. The Cu(II), Ni(II) and Zn(II) solutions were obtained by respectively dissolving Cu(ClO₄)₂·5H₂O, Ni(ClO₄)₂·6H₂O, and Zn(CH₃COO)₂·2H₂O in water. The metal concentrations ([Cu(II)] = 2.50 × 10⁻² M, [Ni(II)] = 2.47 × 10⁻² M and [Zn(II)] = 2.22 × 10⁻² M) were determined by titration with ethylenediaminetetraacetic acid (EDTA)¹⁰. Doubly distilled water passed through a Millipore Q reagent water system (Millipore Co.) was used throughout. Freshly prepared solutions of AHTC 1 × 10⁻⁴ M, of ionic strength I = 0.1M KCl, buffered at pH 7.2 (with HEPES buffer [4-(2-hydroxyethyl)-1 piperazineethanesulfonic acid]) were used for the spectrometric titrations.

The absorption as well as the CD spectra were recorded by keeping the ligand concentration constant and by varying that of the metal ion. Several sets of similar experiments were carried out. The concentration stability constants $\beta_{pqr} = [M_p H_q A_r] / [M]^p [A]^q [H]^r$ (A = ligand) were calculated from the absorption titration curves by means of the SQUAD program¹¹, by analyzing at least three titration experiments separately. The species distributions against the total metal concentration were determined by the SCECS program¹².



Scheme 1.

Results and Discussion

In a previous report⁸ we determined the acidity constants of AHTC hydrochloride in aqueous solution using a potentiometric method. Three pK_a were obtained: pK_{a1} = 3.23 ± 0.08, pK_{a2} = 5.94 ± 0.09 and pK_{a3} = 8.48 ± 0.02 (I = 0.1 M KCl; 25 °C). The totally protonated species with a positive charge located on the dimethylammonium group was represented by LH₃⁺. The first deprotonation was assigned to the enolic proton at C3, yielding the neutral zwitterionic form, LH₂, in which the positive charge is located on the dimethylammonium group and the negative charge delocalized over the O1-C1-C2-C3-O3 system on ring A. The second pK_a was attributed to the phenolic proton at C10 with formation of LH⁻, and the third one was ascribed to the dimethylammonium group, leading to the totally deprotonated L²⁻ species.

According to Harada *et al.*¹³, the UV-visible absorption spectra of aqueous solutions of AHTC (0.1 M KCl) exhibit four bands at 428, 335, 270 and 224 nm, which can be attributed to the ¹A → ¹L_b, ¹A → ¹L_a, ¹A → ¹B_b and ¹A → ¹B_a transitions of the BCD ring chromophore. In addition, the band at 270 nm contains a contribution from the A-ring chromophore.

The CD spectral variations of an aqueous solution of AHTC with pH were recently reported by us⁹. Figure 1 shows the spectra at pH 1, 4.6, 7 and 10, in which the respective LH₃⁺, LH₂, LH⁻ and L²⁻ species are present at more than 90%. All CD curves show two negative bands around 242 and 428 nm. However, great differences are observed in the 260–290 nm region. LH₃⁺ and LH⁻ display one positive absorption at 270 nm, whereas a CD signal of the couplet type centered at 272 nm, with a positive absorption at 265 nm and a negative one at 288 nm is observed in the spectrum of LH₂. This species

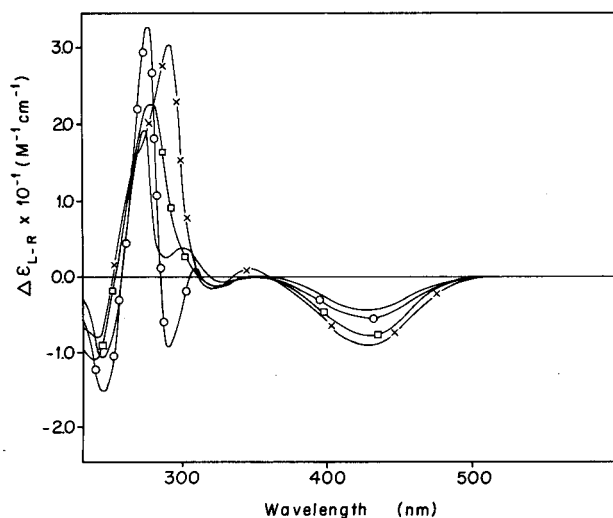


Figure 1. CD spectra of an aqueous solution of AHTC 1×10^{-4} M (I = 0.1M KCl) at different pH: pH 1.5 (—), pH 4.9 (---), pH 7.1 (—□—), and pH 10.7 (---x---).

probably adopts the so-called “twisted” conformation for tetracycline¹⁴, in which N4 is hydrogen bonded to O3. In this conformation C1, C2, C3 and Camide lie above the BCD rings, and the dimethylammonium group projects away from the rest of the molecule. As we previously suggested⁹, in the LH₂ species this arrangement probably allows for the occurrence of a chiral exciton coupling between two very intense $\pi \rightarrow \pi^*$ transitions of the BCD and A chromophoric regions, responsible for the double Cotton effects at 272 nm. In the L²⁻ species, N4 is deprotonated, hydrogen bonding occurring between N4 and O12a by an extension of the A ring, into what has been designated the “extended” conformation¹⁴. The spectrum of L²⁻ shows a positive absorption at 284 nm with a shoulder at 270 nm, the couplet pattern no longer being observed, probably as a consequence of a change in the chirality of the A ring chromophore by the adoption of the “extended” conformation¹⁵.

As stated by other authors in relation to TC¹⁴, we may assume that one structural arrangement is found in acidic to neutral media and another more “extended” conformation in basic solutions. Therefore, as for TC, the arrangements of LH₃⁺, LH₂ and LH⁻ are not supposed to be very different. However, in the zwitterion the twisting of the molecule particularly favors the occurrence of a coupling of the BCD and A chromophores, leading to an intense split type CD signal not observable in the spectra of LH₃⁺ and LH⁻.

Ni(II)-AHTC interaction in aqueous solution

At pH7, in the absence of the metal, the main species present in the solution is LH⁻⁹. By the addition of increasing concentrations of Ni(II) to a 1×10^{-4} M solution of AHTC in molar ratios [Ni(II)]/[AHTC] ranging from 0 to 1 the band at 428 nm shifts to 445 nm in the absorption spectrum, and to 450 nm in the CD spectrum (Fig. 2), with

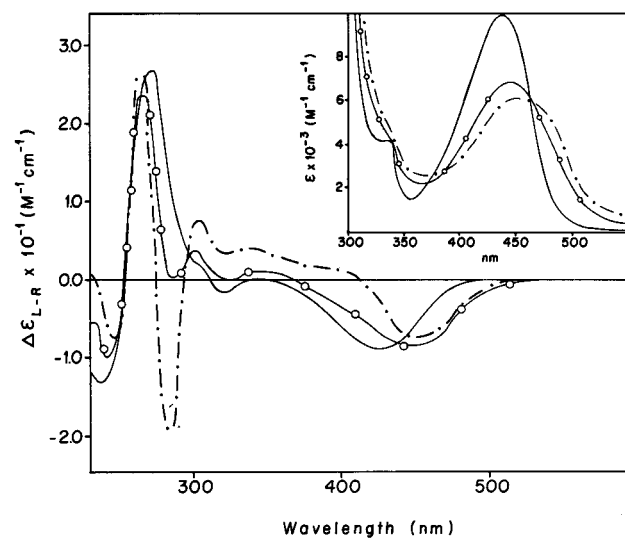


Figure 2. CD and absorption (inset) spectra of an aqueous buffered solution of 1×10^{-4} M AHTC (—) at pH 7 after the addition of Ni(II) ions at different molar ratios [Ni(II)]/[AHTC]: 0.4 (---) and 1.0 (—•—).

the appearance of a shoulder at 475 nm in both spectra. The shifting of the visible absorption indicates coordination of the metal to the BCD chromophore, probably at O11 and O12 as we proposed in a previous report⁸ for the Ni(II)-AHTC complex in the solid state.

In the presence of the metal, the CD spectrum of AHTC undergoes striking variations in the 260 - 300 nm region (see Fig. 2). In fact, the absorption at 270 nm splits into a positive peak at 264 nm and a negative one at 285 nm, and the appearance of the couplet type CD signal, is observed characteristic of the LH₂ form.

The absorbance data in the visible range were used in the calculation of the complex formation constant by means of the SQUAD program¹¹. Fifty absorbances were measured per spectrum. The best fitting results were obtained by supposing the formation of only the MH₂L₂ species. We found $\log \beta_{\text{MH}_2\text{L}_2} = 25.59 \pm 0.08$ (see Table 1). The experimental (Fig. 2) and calculated (not shown) spectra were in excellent agreement. The species distribution in solution against the total metal concentration, represented in Fig. 3, shows that at $[\text{Ni(II)}]/[\text{AHTC}] = 1$, 62% of the ligand is present as the MH₂L₂ complex.

Cu(II)-AHTC interaction in aqueous solution

By addition of increasing concentrations of Cu(II) to a constant (1×10^{-4} M) concentration of AHTC in molar ratios $[\text{Cu(II)}]/[\text{AHTC}]$ ranging from 0 to 1.3 at pH 7, the band at 428 nm shifts to 445 nm in the absorption spectrum and to 480 nm in the CD spectrum, with the appearance of a shoulder at 490 nm in both spectra (Fig. 4). The shifting of the absorption in the visible range suggests that the metal coordinates to the BCD chromophore, probably at O11 and O12, as we have previously proposed⁸ for the Cu(II) - AHTC complex in the solid.

In the 260-300 nm region the intensity of the CD maximum at 270 nm diminishes and the appearance of a

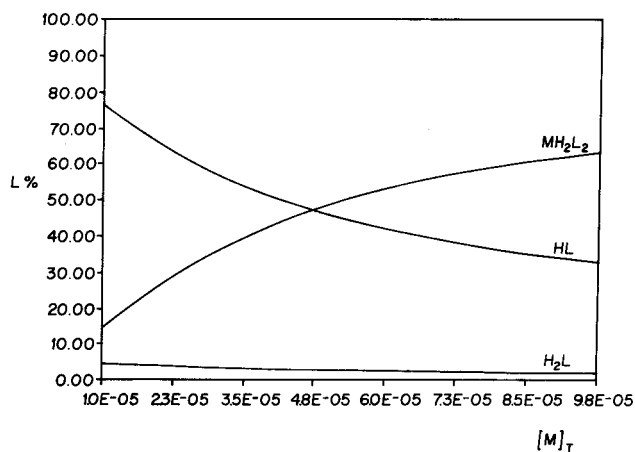


Figure 3. Species distribution of the system Ni(II) - AHTC at pH 7 in the experiment of Fig. 2.

positive band at 300 nm is observed. Two isodichroic points at 290 and 454 nm are noticeable.

As in the previous case, the absorbance data in the visible range were used in the calculation of the complex formation constant by means of the SQUAD program¹¹. The best fitting results were obtained by supposing the formation of only MHL. For the sake of clarity charges were omitted in this text. The experimental (Fig. 4) and calculated (not shown) spectra of the complex were in excellent agreement. We found $\log \beta_{\text{MHL}} = 12.12 \pm 0.02$ (Table 1). The species distribution in solution against the total metal concentration (not shown) indicates that at $[\text{Cu(II)}]/[\text{AHTC}] = 1.3$ only 33% of the ligand is present as the MHL complex. For higher metal concentrations precipitation occurs.

Table 1. Formation Constants of the proton and metal - AHTC systems.

System	Complex Stoichiometry	Log $\beta^{(1)}$
Proton-AHTC	LH ⁺	8.48 ± 0.02
	LH ₂	14.42 ± 0.09
	LH ₃ ⁺	17.7 ± 0.2
Ni(II)-AHTC	MH ₂ L ₂	25.59 ± 0.08
Cu(II)-AHTC	MHL	12.12 ± 0.02
	MHL	15.59 ± 0.01
Zn(II)-AHTC	MHL	16.15 ± 0.10
	M ₂ L	15.69 ± 0.13

(1) Values obtained by analyzing at least three experiments separately. In all cases the sum of the deviation squares of at least 10 solutions and 50 wavelengths was inferior to 5×10^{-3} .

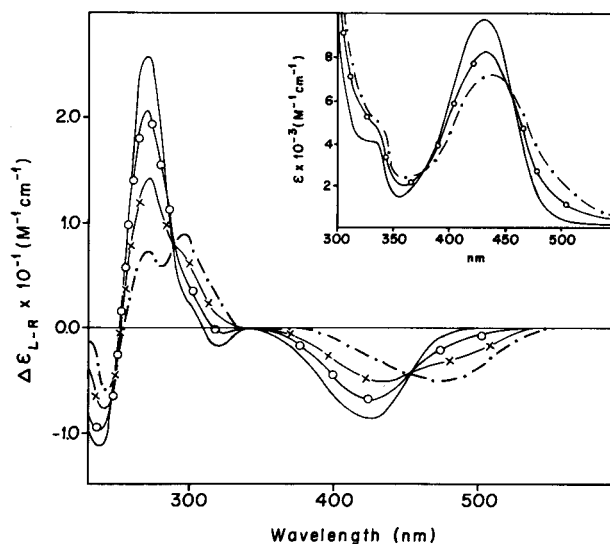


Figure 4. CD and absorption (inset) spectra of an aqueous buffered solution of 1×10^{-4} M AHTC (—) at pH 7 after the addition of Cu(II) ions at different molar ratios $[\text{Cu(II)}]:[\text{AHTC}]$: 0.4 (○), 0.8 (×) and 1.3 (●).

Zn(II) - AHTC interaction in aqueous solution

At pH 7, by the addition of increasing concentrations of Zn(II) to a constant (1×10^{-4} M) concentration of AHTC in molar ratios $[\text{Zn(II)}]/[\text{AHTC}]$ ranging from 0 to 3, the maximum at 428 nm shifts to 440 nm in the absorption spectrum and to 458 nm in the CD spectrum (see Fig. 5), indicating coordination of the metal ion to the chromophore of the BCD rings probably at O11 and O12, as we proposed for the previous cases.

In the 260-300 nm region, the band at 270 nm shifts to 262 nm, and the appearance of a negative absorption at 284 nm for high metal concentrations is observed. The final split type CD pattern is similar to that of LH₂, the intensity of the bands being lower in the spectrum of the complex.

The absorbance data in the visible range used in the calculation of the complex formation constants by means of the SQUAD program¹¹ were fitted by supposing the formation of the MHL and MOH^+ species. In fact, at pH 7 the concentration of the hydrolyzed MOH^+ species is significant, as may be inferred from the value of the hydrolysis constant of Zn(II) ($\log \beta = -4.10$)¹⁶. We found $\log \beta_{\text{MHL}} = 15.59 \pm 0.01$. The experimental (Fig. 5) and calculated (Fig. 6a) spectra were in excellent agreement. The species distribution against the total metal concentration (Fig. 7a) shows that at $[\text{Zn(II)}]/[\text{AHTC}] = 3$ only 55% of the ligand is present as the MHL complex.

The possibility of the formation of the M₂L complex in addition to MHL and MOH^+ , by coordination of a second Zn(II) ion to the N4 and O3 positions on ring A was also considered (see Discussion), giving $\log \beta_{\text{MHL}} = 16.15 \pm 0.10$ and $\log \beta_{\text{M}_2\text{L}} = 15.69 \pm 0.13$ (Table 1). The experimental (Fig. 5) and calculated (Fig. 6b) spectra were

also in excellent agreement with this assumption. The species distribution against the total metal concentration, shown in Fig. 7b, indicates that in the presence of a large excess of Zn(II) the M₂L species predominates.

Finally, taking into consideration the presence of acetate (Ac) in solution (from the Zn(II) salt), the formation of the M(HL)Ac species along with MOH^+ , was considered and convergence was achieved. Although the fit was not as good as in the previous cases, this possibility cannot be discarded. However, convergence was not achieved when the presence of both MHL and M(HL)Ac, along with MOH^+ , was considered, suggesting that the assumption of

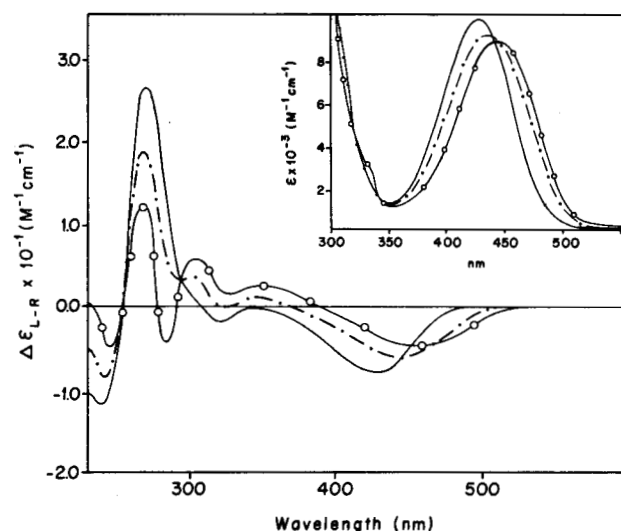


Figure 5. CD and absorption (inset) spectra of an aqueous buffered solution of 1×10^{-4} M AHTC (—) at pH 7 after the addition of Zn(II) ions at different molar ratios $[\text{Zn(II)}]:[\text{AHTC}]$: 0.2 (---) and 3 (·-·).

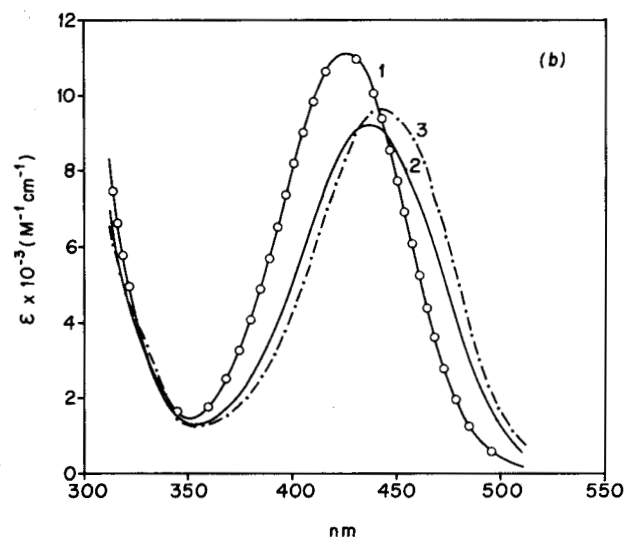
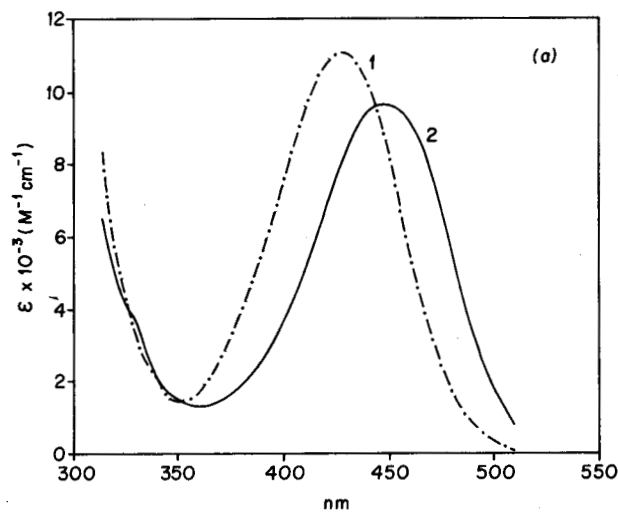


Figure 6. Calculated absorption spectra of AHTC and its Zn(II) complexes by supposing the presence of: (a) HL (1) and MHL (2); (b) HL (1), MHL (2) and M₂L (3).

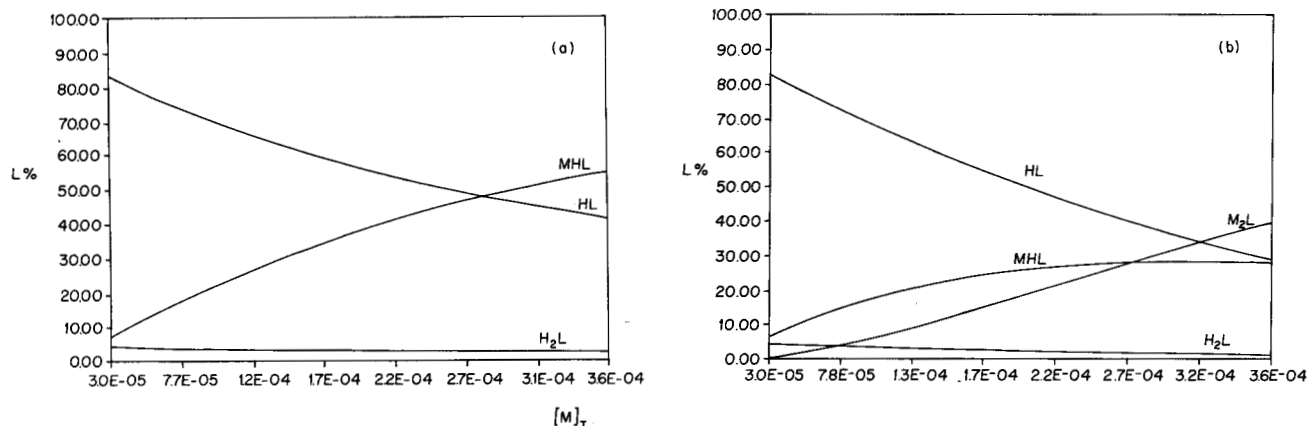


Figure 7. Species distribution of the system Zn(II)-AHTC at pH 7 in the experiment of Fig. 5 by supposing the presence of (a) only the MHL species, and (b) the MHL and M_2L species.

formation of MHL is probably more reasonable. Moreover, in spite of the high value of the formation constant of the ternary $M(HL)Ac$ species in relation to that of MHL, the program did not eliminate the latter, indicating that the presence of MHL must be considered.

Discussion

We suggest that two AHTC molecules coordinate Ni(II) through the phenolate and carbonyl oxygens O11 and O12, respectively, forming a MH_2L_2 species. As may be inferred from the couplet type CD pattern, in the Ni(II) complex the ligands probably adopt the conformation of the zwitterion (LH_2). As stated by other authors in relation to TC¹⁷, in the "twisted" conformation of LH_2 , the juxtaposition of O1, O12 and O11 provides an additional chelating site. In this case, two AHTC ligands would be tridentately attached to the metal in an octahedral arrangement. Moreover, we suggest the occurrence of a chiral exciton coupling of two intense $\pi \rightarrow \pi^*$ transitions of the BCD and A chromophores, either on the same ligand molecule or on two distinct ligand molecules.

For the Zn(II) - AHTC interaction we propose that the metal ion coordinates to O11 and O12, forming the MHL species. Furthermore, coordination to Zn(II) probably induces the ligand to adopt the conformation of LH_2 , as may be inferred from the split type CD pattern. The high value of the complex formation constant suggests that AHTC probably acts as a tridentate system. The geometry of the complex is probably tetrahedral, a water molecule occupying the fourth coordination site. In a previous work⁹ we demonstrated that Mg(II) also forms a MHL complex. In the presence of a large excess of metal, the M_2L species is also formed by the attachment of a second magnesium ion to the N4 and O3 positions on ring A, with the appearance of a double Cotton effect in the CD spectrum. The possibility of the formation of a M_2L complex by coordination of a second Zn(II) ion to the same sites on ring A cannot be

excluded. In the presence of a large excess of metal, the Mg(II) M_2L complex displays a very intense couplet type CD signal at 272 nm⁹, whereas the addition of a large excess of Zn(II) is prevented by precipitation. As is well known, there are certain resemblances between Mg(II) and Zn(II) that are related to size and charge phenomena¹⁸. In fact, the ionic radii of these cations are similar¹⁸, the capacity for forming M_2L possibly being related to the ability of both metal ions to attach to the N4 - O3 sites of the ligand.

Regarding the complexation of AHTC to Cu(II), we propose that the only species formed is MHL, in which AHTC acts as a bidentate ligand which coordinates the metal through O11 and O12. The complex geometry is most probably a distorted octahedron, the remaining binding sites being occupied by water molecules. In this case, no couplet type CD signal was observed, indicating that the complex conformation is probably different from that of the zwitterion, the ligand probably being bidentate and adopting a more extended conformation.

At this stage some comments must be made concerning the values of the complex stability constants. The stability constant of the Cu(II) MHL complex seems too low compared to those of the Ni(II) MH_2L_2 and Zn(II) MHL complexes (Table 1), probably due to the lower affinity of copper for oxygen. In addition, in the cases of both the Ni(II) and Zn(II) complexes, the split type CD pattern suggests that the ligand adopts the conformation of the zwitterion LH_2 , in which AHTC can act as a tridentate ligand, able to form more stable compounds. The unexpectedly high value of the Zn(II) MHL complex formation constant is probably due to the higher affinity of the metal for oxygen, as well as to the tridentate chelation of AHTC in this compound.

Conclusions

The results indicate that at pH 7 AHTC coordinates Ni(II), Cu(II) and Zn(II) through the O11 and O12 positions

of the system of the BCD rings. In the Ni(II) and Zn(II) complexes the ligand is probably tridentate, adopting the same conformation as the zwitterion (LH₂), in which the oxygen O1 is the third coordination site. In the Cu(II) complex, only the oxygens O11 and O12 are attached to the metal, the conformation of the ligand being different from that of LH₂.

Depending on the concentration of AHTC resulting from the ingestion of degraded TC, the interactions of AHTC with Cu(II) and Zn(II) may be sufficiently significant to influence the toxic side effects of the antibiotic. Furthermore, the distinct conformational behavior of AHTC in the presence of Cu(II) and Zn(II), as well as the difference in stability of the AHTC complexes with these cations may also have biological consequences.

Acknowledgments

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

We are indebted to Professor A. Garnier-Suillerot for the CD facilities at the Laboratoire de Chimie Bioinorganique, Université Paris XIII.

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