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# Copper(II) complexes of methimazole, an anti Grave's disease drug. Synthesis, characterization and its potential biological behavior as alkaline phosphatase inhibitor

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**Abstract** Methimazole (MeimzH) is an anti-thyroid drug and the first choice for patients with Grave's disease. Two new copper(II) complexes of this drug: $[Cu(MeimzH)_2(NO_3)_2].0.5H_2O$ and $[Cu(MeimzH)_2(H_2O)_2](NO_3)_2{\cdot}H_2O$ were synthesized and characterized by elemental analysis, dissolution behavior, thermogravimetric analysis and UV-vis, diffuse reflectance, FTIR and EPR spectroscopies. As it is known that copper(II) cation can act as an inhibitor of alkaline phosphatase (ALP), the inhibitory effect of methimazole and its copper(II) complexes on ALP activity has also been investigated.

**Keywords** Methimazole · Copper · Biometals · Grave's disease · EPR · FTIR · UV-visible · ALP inhibition

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## Introduction

Methimazole (MeimzH) is an antithyroid drug commonly used to treat Graves' disease which cause thyrotoxicosis characterized by goiter, hyperthyroidism, eye disease, etc. (Tene et al. 2001). This drug reduces the excessive thyroid activity acting as an inhibitor of the enzyme thyroid peroxidase by blocking the synthesis of thyroid hormone. The therapeutic efficacy of single daily doses of methimazole has been proved to be better than propylthiouracil in the induction of euthyroidism (Chih-Tsueng et al. 2004). Several investigations related to different aspects of methimazole have been published. Medicinal research includes: (a) effects on stress prevention and chemical induced gastropathy in rats (Al Moutaery 2003), (b) the inhibition of melanin synthesis in cultured B16 melanocytes (Kasraee et al. 2004), (c) the correction of insulin resistance in methimazole-treated patients (Tene et al. 2001), (d) the upregulation of the T-cell-derived cytokines (Kocjan et al. 2004), (e) the effect of antioxidant supplementation on superoxide dismutase (SOD) activity (Bacic-Vrca et al. 2005), among others. It is well known that metal ions play a vital role in a vast of widely different biological processes and most of the biologically active compounds act via chelation. Inorganic and bioinorganic compounds, as well as their applications in medicine, have attracted the attention of scientists in order to advance in the investigations on cancer chemotherapy and chemoprevention, antiarthritis

compounds and antimicrobials agents (Williams 1972; Cameron 2001; Bakhtiar and Ochiai 1999; Taylor and Williams 1995). By using methimazole as a chelating ligand, several metal-coordination complexes have been synthesized (Raper 1996, 1997; Raper and Brooks 1997) including mercury compounds as potential protective agents in organomercurial intoxication in the detoxification area (Buncel et al. 1982). Although some copper(I) and copper(I)-copper(II) complexes of methimazole have been studied (Raper 1996, 1997), to our knowledge only one unstable copper (II) complex has been reported in the literature and no enzymatic activity has been reported.

Focus in the last point, our work is intended to study aspects related to unknown potential activities of methimazole and its copper complexes.

The biological action of the alkaline phosphatase is well documented. The ALP in serum is associated with metabolic bone (Hypophosphatasia) and liver diseases and also is used as a marker of osteoblastic differentiation (Li et al. 2008; Whyte (2007). Particular attention was given to the inhibition of the enzymatic activity, and several investigations with vanadium compounds have been performed both in vitro and in vivo. It was considered that the ability of these compounds to inhibit ALP activity was a possible mechanism of action in their mimetic insulin effect and other physiological behavior (Cortizo et al. 1994; Etcheverry et al. 2001; Li et al. 2008; Ferrer et al. 2006; Etcheverry and Cortizo 1998). Related to inhibition of tyrosine phosphatases some complexes of other transition metals were tested as insulin mimetic compounds (Redher 2003; Thompson et al. 1999). Associated to the methimazole, insulin sensitivity increased significantly after methimazole treatment for the correction of the hyperthyroidism in Graves' disease (Tene et al. 2001), as well as by the use of antioxidants (Diplock 1991).

In this exploration we found a report in which alkaline phosphatase activity was inhibited by copper in phytoplankton cultures, cell-free enzyme preparations, and natural populations of phytoplankton. In this, copper toxicity correlated to the net phosphorus nutrition of phytoplankton as it was proven by blocking sources and phosphate cycles mediated by alkaline phosphatase (Rueter 1983). Strong inhibition of the activity of a variety of hydrolases has been reported in metal polluted soils over the past years. The effects of copper on the activity and kinetics of free and immobilized acid phosphatase were described (Huang and Shindo 2000). In this study, it has been reported that the better susceptibility of the enzyme to Cu(II) at higher pH is attributed to the deprotonation of the amino acids of enzyme protein that facilitate its interactions with the metal ion. In addition, a higher residual activity of the enzyme for copper citrate than for copper chloride systems was accredited to the lower affinity of acid phosphatase to Cu in the former molecule. In the aim to study the potential use of enzyme activities as biochemical indicators of soil pollution and soil quality, the effects of Cd on the $ED_{50}$ (ecological dose) of acid and alkaline phosphatase activities of contrasting soils with or without Cu and/or Zn have been studied. These studies suggest that the presence of copper increased the toxic effect of Cd in soil by reducing phosphatase activities (Renella et al. 2003). On the contrary, in a previous work (Martin and Evans 1991) the effect of the Inositol hexaphosphate-Cu(II) complex in solution on the Intestinal Alkaline Phosphatase activity was reported in order to investigate the introduction of Cu(II) in the active site of Zn-containing enzyme. They established that in absence of Cu(II) the inhibition was 40%, the activity decrease to only about 5% when Cu(II) and ligand are present at a ratio of 6.2:1 and that Cu(II) alone had no effect on the enzyme activity. These findings point our attention to examine the effects of methimazole and two new copper(II) complexes of this drug on alkaline phosphatase activity, and to reinvestigate the effect of Cu(II).

The synthesis, chemical characterization and dissolution behavior of two new copper(II) complexes of methimazole are described. Besides, the effect on ALP activity of the parental drug and its two copper(II) complexes are also reported.

## Materials and methods

### Reagents and instrumentation

All chemicals were of analytical grade. Copper(II) nitrate trihydrate was obtained from Merck, Methimazole, para-nitrophenyl phosphate (p-NPP), bovine intestinal ALP, anhydrous lactose and all the other analytical grade chemicals used were purchased from Sigma.

FTIR spectra of powdered samples were measured with a Bruker IFS 66 FTIR-spectrophotometer from 4000 to 400 $cm^{-1}$ in the form of pressed KBr pellets. Electronic absorption spectra were recorded on a Hewlett-Packard 8453 diode-array spectrophotometer, using 1 cm quartz cells. Diffuse reflectance spectra were registered with a Shimadzu UV-300 instrument, using MgO as an internal standard. Elemental analyses for carbon, hydrogen and sulfur were performed using a Carlo Erba EA 1108 analyzer. Thermogravimetric (TG) and differential thermal analysis (DTA) were performed on a Shimadzu system (models TG-50 and DTA-50, respectively), working in an oxygen flow (50 ml/min) at a heating rate of 10°C/min. Sample quantities ranged between 10 and 20 mg. $Al_2O_3$ was used as a DTA standard. For dissolution capacity test: Hanson Research SR6 (Apparatus 2-Paddle Apparatus) equipment and Spectrophotometer Metrolab 1700. A Bruker ESP300 spectrometer operating at X and Q- bands and equipped with standard Oxford low temperature devices was used to record the spectra of the compounds at different temperatures. The magnetic field was measured with a Bruker BNM 200 gaussmeter, and the frequency inside the cavity was determined by using a Hewlett-Packard 5352B microwave frequency counter. A computer simulation of the EPR spectra was performed using the programs SimFonia (WINEPR SimFonia 1996). Base line corrections, normalization, curve-fitting and calculations were carried out by means of Grams/32 (Galactic Industries Corporation, USA) software.

## Preparative

### *[Cu(MeimzH)$_2$(NO$_3$)$_2$].0.5H$_2$O*

Copper nitrate trihydrate (1 mmol, 0.170 g) dissolved in ethanol (10 ml) was added to an ethanolic solution (10 ml) of methimazole (MeimzH) (2 mmol, 0.228 g) under continuous stirring. Then, the pH of the solution was adjusted to 9 by adding small aliquots of sodium methoxide solution. The resulting mixture was stirred at room temperature for 20 min and the resulting violet colloidal product was separated by centrifugation. After that, it was washed several times with ethanol and dried in vacuo. *Anal.* Calc. for $C_8H_{13}N_6S_2O_{6.5}Cu$: C, 22.61%; H, 2.82%; N, 19.78%; S, 15.08%. Found: 23.00%; H, 2.70%; N, 20.21%; S, 15.6%. Yield: 75%. The thermogravimetric analysis (TGA) carried out under an $O_2$ atmosphere-50 ml/min, heating rate of 10°C/min, in the 25–1000°C range, showed two clearly defined steps: (1) 25–50°C ($\Delta\omega_{exp} = 2.03\%$ y $\Delta\omega_{calcd} = 2.12\%$, DTA = 41°C, endo), loss of hydratation $H_2O$, (2) combustion of the compound to CuO (residual mass $\Delta\omega = 19.18\%$ of the compound, supported by FTIR spectroscopy).

### *[Cu(MeimzH)$_2$(H$_2$O)$_2$](NO$_3$)$_2$·H$_2$O*

This compound was prepared by a similar procedure to that described above, except that water was used as a solvent and the pH was adjusted to 8 with NaOH 1 M. The blue powder immediately formed, was filtered off, and the product was washed several times with distilled water. *Anal.* Calc. for $C_8H_{18}N_6S_2O_9Cu$: C, 20.45%; H, 3.4%; N, 17.89%; S, 13.63%. Found: 23.00%; H, 2.70%; N,17.5%; S, 13.8. %. Yield: 82%. TGA ($O_2$ atmosphere-50 ml/min, heating rate of 10°C/min, 25–1000°C range) showed the following processes: (1) 25–100°C, loss of one $H_2O$ molecule ($\Delta\omega_t = 3.83\%$, $\Delta\omega_{exp} = 3.85\%$, DTA = 63°C, endo), (2) a second step between 100–200°C, indicating the lost of the two other water molecules ($\Delta\omega_t = 11.5\%$, $\Delta\omega_{exp} = 10.80\%$, DTA = 173.5°C, endo), (3) complete thermal decomposition of the sample and formation of CuO (anhydrous sample, $\Delta\omega = 17.07\%$, FTIR spectroscopy).

## Dissolution profiles of the complexes

The dissolution profiles were performed according to the United States Pharmacopeia (USP 30) (U.S. Pharmacopeia 2007). Capsules No 3 were prepared with homogenous mixture (20 mg of the complex and 130 mg of anhydrous lactose) in accord to methimazol formulation (Abdou 1989). Dissolution testing of capsules was performed in distilled water (pH 5–6), HCl 0.1 M, sodium lauryl sulfate 0.01 M and buffer $KH_2PO_4$ (pH = 6.8, 0.2 M). The dissolution medium was 500 ml at 37°C, stirred at 50 rpm and 100 rpm. For dissolution profiles, 10.0 ml aliquots were withdrawn at 5; 10; 20; 30 and 40 min. The solutions were immediately filtered off through 0.45 μm nylon filter. One milliliter of the filtered aliquots was pipetted into 10 ml volumetric flask for [Cu(MeimzH)$_2$(NO$_3$)$_2$].0.5H$_2$O and into 25 ml

volumetric flask for $[Cu(MeimzH)_2(H_2O)_2](NO_3)_2 \cdot H_2O$ with fresh dissolution fluids in each case. Drug release (DR%) was assayed by electronic spectroscopy, UV absorbance was measured at 215 nm. Cumulative percentages of the dissolved drug from the tablets were calculated and plotted versus time.

### Stability studies

In order to determine the stability of the compounds during the preparation of the solutions for the enzymatic measurements, the variation of the UV-vis spectra with time was performed. Because of the low solubility of the complexes in water, the dissolution has been carried out in hot DMSO (dimethylsulfoxide). The copper(II) d-d electronic absorption bands in the visible spectral range were monitored.

### Alkaline phosphatase specific activity

The effect of methimazole, copper(II) cation and copper(II) complexes of methimazole on ALP activity was determined by UV-vis spectroscopy. The reaction was started by the addition of the substrate para-nitrophenyl phosphate (p-NPP) and the product p-nitrophenol was monitored by the absorbance changes at 405 nm. Briefly, the experimental conditions for ALP specific activity measurement were as follows: 1 μg/ml of bovine intestinal ALP and 5 mM of p-NPP were dissolved in the incubation buffer (55 mM glycine + 0.55 mM $MgCl_2$, pH = 10.5) and held for 10 min. The effects of the compounds were determined by addition of different concentrations (1–100 μM) of each one to the pre-incubated mixture. The solutions of the complexes were prepared in DMSO before adding the buffer to obtain the desired final concentrations. The effect of each concentration was tested at least in triplicate in three different experiments.

## Results and discussion

### FTIR spectroscopy

The ligand, a heterocyclic thione, methimazole, presents thione-thiol tautomerism in the molecule. The thione tautomer is the dominant species in the solid state. In solution, several factors including pH, the nature of the metal and the solvent affect the equilibrium and the thiol form became the predominant species (Raper et al. 1991). The prevalence of the thione form is confirmed by the absence of the $\nu$(SH) band at ca. 2500 $cm^{-1}$ and the presence of the $\nu$(NH) band at 3108 $cm^{-1}$ (Table 1) (Raper 1997). In coordination compounds involving thioamide group, four bands are characteristic in the infrared spectra. These bands appear in the following regions 1570–1395 $cm^{-1}$, 1420–1260, 1140–940 and 800–700 $cm^{-1}$ corresponding to the called "thioamide I, II, III, IV bands" (Jolley et al. 2000). Bands I and II have a little C=S contribution and originate mainly in C=N and NH deformation modes. On the contrary, bands III and IV have a significant C=S content. These bands are used to predict the mode of coordination of heterocyclic thione complexes. The lack of interaction via the nitrogen is supported for the insignificant changes in the position of the $\nu$(NH) band in the complexes (Table 1). Also, no positive shifts are observed for the thioamide I band which is characteristics for M–N coordination. The most important changes are observed in the thioamide IV band which has the largest proportion of $\nu$ (CS). The effect that produces the thione donation consists in the displacement of this band to lower wavenumbers (Bell et al. 2004, Raper et al. 1998).

Bands corresponding to the vibrational modes of nitrate ion were also detected. One intense band located at 1380 $cm^{-1}$ assigned to a combination mode band ($\nu_1 + \nu_4$) is observed for the blue complex together with the band related to an in plane bending vibration ($\nu_4$) which appears at 684 $cm^{-1}$. This suggests the presence of ionic nitrate group for this complex (Battaglia et al. 1976). In the spectra of the violet complex the splitting of $\nu_4$ Into two bands (695 and 682 $cm^{-1}$) is consistent with the presence of a monodentate nitrate group (Pettinari et al. 1999). The difference on these two values, $\Delta = 13$ $cm^{-1}$, confirms the interaction of a single oxygen atom of nitrate anion with copper(II) (Nakamoto 1986). The others nitrate bands are hidden under ligand absorption bands.

### Diffuse reflectance and UV-vis spectra

The diffuse reflectance spectrum of $[Cu(MeimzH)_2(NO_3)_2].0.5H_2O$ showed two bands at 760 and 560 nm while the spectrum of $[Cu(MeimzH)_2(H_2O)_2](NO_3)_2 \cdot H_2O$ showed a broad band with

**Table 1** Assignments of some characteristic FTIR bands ($cm^{-1}$) of methimazole, $[Cu(MeimzH)_2(NO_3)_2].0.5H_2O$ and $[Cu(MeimzH)_2(H_2O)_2](NO_3)_2{\cdot}H_2O$

| MeimzH | $[Cu(MeimzH)_2(NO_3)_2].0.5H_2O$ (violet) | $[Cu(MeimzH)_2(H_2O)_2](NO_3)_2{\cdot}H_2O$ (blue) | Assignments | |
|---|---|---|---|---|
| 3108 s | 3111 s | 3120 s | $\nu$ (NH) | |
| 1462 s | 1460 s | 1459 s | I | Thioamide bands |
| 1271 s | 1284 s | 1279 s | II | |
| 1084 m | 1086 m | 1086 m | III | |
| 766 m | 731 m | 739 m | IV | |
| | | 1380 s | $(\nu_1 + \nu_4)$ | Ionic $NO_3^-$ group |
| | | 684 m | $\nu_4$ | |
| | 695,682 w | | $\nu_4$ | Unidentate $ONO_2^-$ |

*s* strong, *m* medium, *w* weak

a maximum at 750 nm. In both, the bands observed at low energy were assigned to the d-d transitions of the copper(II) ion in square coplanar environment (450–830 nm region, rather asymmetric due to $d^9$ configuration Jahn–Teller effect) (Lever 1984).

In solution the UV-visible spectrum of the $[Cu(MeimzH)_2(H_2O)_2](NO_3)_2{\cdot}H_2O$ complex in ethanol displayed a broad band with maximum at 640 nm ($\varepsilon = 1002.6\ M^{-1}\ cm^{-1}$) The observed band probably are related to significant solvent effects and the appearance of intense electronic spectral bands also may be attributed to the contribution of charge transfer S → Cu(II) transitions (Ainscough et al. 1989).

## Stability studies

The complexes were dissolved in hot DMSO in order to evaluate the stability in similar conditions to those of the in vitro studies. The conversion of violet and the blue compound in a single predominant solution species was determined by measuring the variation of the UV-vis spectra with time (15 seg, 5, 10, 15, 25, 50 min). Fig. 1a shows the time-dependent changes in the visible range of the spectra. The electronic spectra show a prominent broad band in the range 500–810 nm. Under these conditions the chemical species that the blue complex gives under dissolution ($[Cu(MeimzH)_2(H_2O)_2](NO_3)_2{\cdot}H_2O$) was stable in time (Fig. 1a B). The violet complex ($[Cu(MeimzH)_2(NO_3)_2].0.5H_2O$) (Fig. 1a A) showed at a broad d-d band with a maximum at 689 nm but after 5 min the initial spectrum changed with formation of a new spectrum comparable to those the blue complex. These results demonstrated that during the manipulation time of the samples for the bioactivity assays both complexes convert to major solution specie with similar coordination mode. Probably, the nitrate ligands interchange with solvent molecules. The retention of the S atom of the MeimzH ligand in the coordination sphere of the metal center is supported taking into consideration the position of the band and the absorptivity values corresponding to a charge transfer process (Ainscough et al. 1987).

In order to identify the nature of the bioactive species, the UV-vis spectra for the violet (Fig 1b) and the blue (Fig. 1c) complexes in DMSO were fitted. We have selected the spectra obtained after 5 min of the dissolution because it is the time elapsed during the dissolution of the complexes previous to the measurements of the enzymatic assays. The best fit showed in both spectra the presence of one band at 676 nm ($\varepsilon = 521\ M^{-1}\ cm^{-1}$, calculated after stabilization) (predominant band), other in a 575–600 range and others in minor percentage probably due to a tiny proportion of the unconverted violet compound. The position and the molar extinction coefficient for the main band lend strong support to attribute the origin of this band to S → Cu(II) charge transfer transition. Similar charge-transfer bands have been assigned in proteins. Blue copper centers are characterized by an intense Cys → Cu(II) LMCT (Cys = cysteine) (ligand-to-metal charge transfer) transition near 600 nm resulting in their blue color. Nevertheless, there is considerable difference in energy between the charge-transfer bands of the copper-metimazol complex and those of the

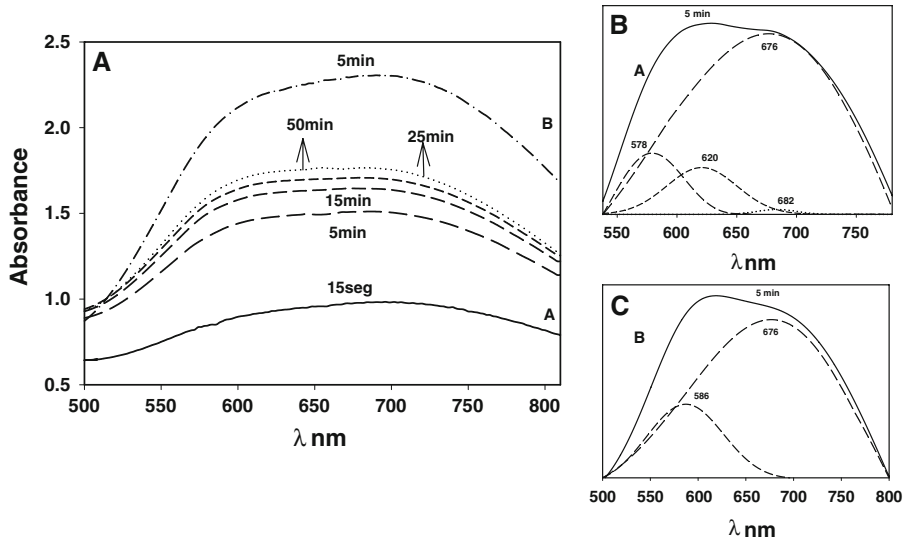


**Fig. 1** **a** *A* UV-vis absorbance spectrum of the [Cu(MeimzH)$_2$(NO$_3$)$_2$].0.5H$_2$O (*violet*) complex at 15 seg (*solid line*), 5 min (*long dash line*), 15 min (*medium dash*), 25 min (*short dash*), 50 min (*dotted line*) in DMSO solvent (4.79 mM). *B* UV-vis absorbance spectrum of the [Cu(MeimzH)$_2$(H$_2$O)$_2$](NO$_3$)$_2$·H$_2$O (*blue*) complex at 5 min in DMSO solvent (4.26 mM). **b** Original UV-vis absorbance spectrum [Cu(MeimzH)$_2$(NO$_3$)$_2$].0.5H$_2$O (*violet*) complex (*solid line*) at 5 min in DMSO solvent and UV-vis absorbance spectrum after curve-fitting (*dash and dotted lines*). **c** Original UV-vis absorbance spectrum [Cu(MeimzH)$_2$(H$_2$O)$_2$](NO$_3$)$_2$·H$_2$O (*blue*) complex at 5 min in DMSO (*solid line*) and UV-vis absorbance spectrum after curve-fitting (*dash lines*)

tetrahedral protein complex (Belle et al. 2005; Mcmillin and Morris 1981).

## Electron spin resonance spectra

The X and Q-band EPR powder spectra of the complexes were measured at room temperature and at 140 K. The polycrystalline sample of [Cu(MeimzH)$_2$(NO$_3$)$_2$].0.5H$_2$O (violet) showed a better resolution in Q-band (Fig. 2a) while X-band could be observed for [Cu(MeimzH)$_2$(H$_2$O)$_2$](NO$_3$)$_2$·H$_2$O (blue) (Fig. 2b). Simulation showed that the violet complex presented $g$-values of $g_1 = 2.065$, $g_2 = 2.10$ and $g_3 = 2.158$ ($g_0 = 2.11$) while the blue complex presented $g$-values of $g_1 = 2.031$, $g_2 = 2.091$ and $g_3 = 2.163$ ($g_0 = 2.095$) which are consistent with rhombic symmetry (Hathaway and Billing 1970). The obtained parameters are in concordance with square-coplanar stereochemistries in a similar $S_2O_2$ donor environment. (Guillon et al. 1998). In order to distinguish the geometry, the $\boldsymbol{R}$ parameter ($\boldsymbol{R} = (g_2 - g_1)/(g_3 - g_2)$) was calculated taking into account that $g_3 > g_2 > g_1$. The calculated $R$ value ($R < 1$) of 0.6 and 0.83, respectively, indicate that the unpaired electron is located mostly on $dx^2 - y^2$ orbital of the copper(II) atom (Naso et al. 2009; Chandra and Gupta 2005). The observed broadening of the resonance line suggests an enhanced magnetic dipole interaction between the paramagnetic centers (Jolley et al. 2000; Bell et al. 2004).

## Dissolution assay

The purpose of the present study was to characterize the dissolution performance of the copper-methimazole complexes in different dissolution media. Figure 3A shows the dissolution profile for [Cu(MeimzH)$_2$(NO$_3$)$_2$].0.5H$_2$O complex using 50 rpm. As can be seen from the figure, better sample dissolution was obtained in water solution in comparison with KH$_2$PO$_4$ and the sodium lauryl sulfate (LSNa) dissolution medium, but the thermodynamic balance is not reached in this case. In contrast, by using HCl solution the percentage of the dissolution increased until a constant value reaching the thermodynamic balance between 30 and 40 min. On the other hand, at 100 rpm the curve shows a better dissolution in KH$_2$PO$_4$ medium, but the equilibrium was obtained in HCl and sodium lauryl sulfate solutions throughout the time course of 30–40 min.

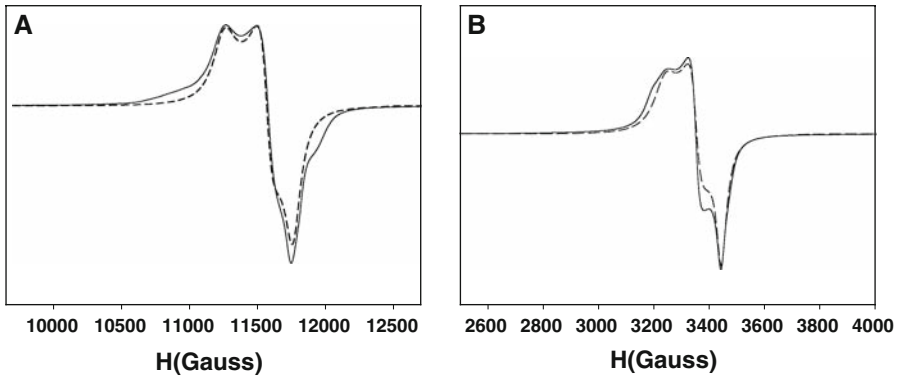


**Fig. 2** **a** EPR powder spectra at 290 K (Q band) of the complex $[Cu(MeimzH)_2(NO_3)_2].0.5\ H_2O$ (*violet*) (*solid line*) and simulated spectrum (WINEPR SimFonia (*dash line*)). **b** EPR powder spectra at 290 K of the complex $[Cu(MeimzH)_2(H_2O)_2](NO_3)_2{\cdot}H_2O$ (*blue*) and simulated spectrum (WINEPR SimFonia)

At 50 rpm and using $KH_2PO_4$ and HCl as dissolution medium, the $[Cu(MeimzH)_2(H_2O)_2](NO_3)_2{\cdot}H_2O$ complex (Fig. 3B) arrived at the thermodynamic balance between 30 and 40 min in spite of the better solubility was obtained in aqueous medium. The drug solubility was improved at 100 rpm in HCl and LSNa solutions reaching the balance after 30–40 min except in the case of $KH_2PO_4$ medium.

It was found that a priori predictions could not be made; the dissolution rate changed as a function of the agitation speed and it was also pH-dependent. In a future other pre-formulation studies have to be

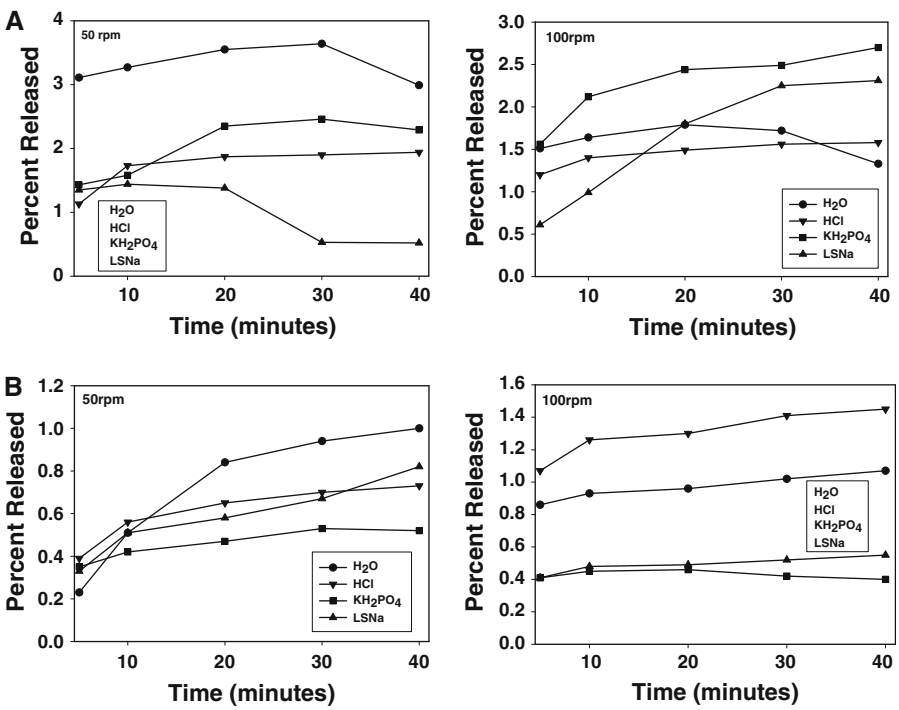


**Fig. 3** Dissolution profiles: **A** $[Cu(MeimzH)_2(NO_3)_2].0.5H_2O$. **B** $[Cu(MeimzH)_2(H_2O)_2](NO_3)_2{\cdot}H_2O$

performed in order to enhance the aqueous solubility and dissolution rate including drug-excipient interactions among others.

## Alkaline phosphatase assays

Alkaline phosphatase (E.C.3.1.3.1) is a metalloenzyme that catalyzes the hydrolysis of phosphate monoesters. The most widely characterized is an 80,000 molecular weight enzyme from *E.coli*, for which a low-resolution X-ray crystal structure is available (Bertini and Luchinat 1983). It is a dimer containing three non-equivalent metal-binding sites in each subunit. Two of them are occupied by zinc ions, one with a catalytic role and the other one with a structural function. The third metal is a Mg(II) cation, also playing a structural role. Alkaline phosphatase is also associated with the surface of algae, specifically the plasmalemma (Rueter 1983).

In Fig. 4 it can be observed the effects of copper(II) cation, the two compounds after 5 min of dissolution in hot DMSO and methimazole on the ALP activity. As it can be seen, the complex ($ED_{50} = 42$ μM) is a stronger inhibitory agent than copper(II) cation in the whole concentration range. Besides, methimazole behaves as a moderate inhibitor ($ED_{50} = 70$ μM) but the complexation with copper improved its activity. Interestingly, our results shows that the copper(II) cation did not display significant inhibitory effect on the ALP activity (an effect of only 10% was observed) in contrast with previous controversial results obtained either in phytoplankton cultures (Rueter 1983) or with the commercial enzyme (Martin et al.). In the former study copper inhibited the enzymatic activity and in the latter investigation the metal ion has no effect on the intestinal alkaline phosphatase. In opposition to our results, the complexation of copper(II) by Inositol hexaphosphate do not improve the inhibitory effect of the ligand on the enzymatic activity (Martin and Evans 1991).

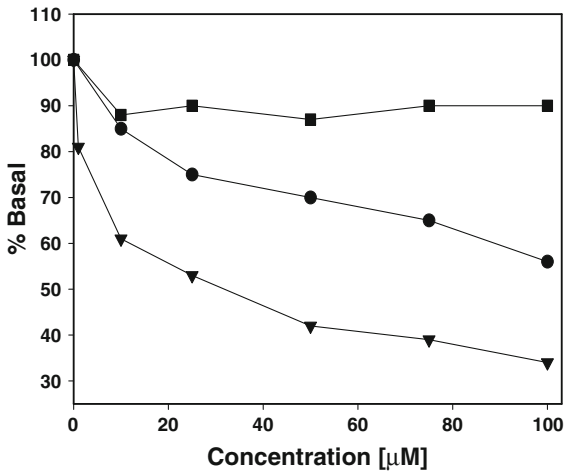


**Fig. 4** Effect of copper(II) ion (*Filled square*), methimazol (*Filled circle*) and the species obtained after dissolution in hot DMSO (5 min), (*Filled inverted triangle*) on ALP activity from bovine intestinal mucose. Initial rate was determined by incubation of the enzyme at 37°C for 10 min in the absence or presence of variable concentrations of the inhibitors. Basal activity was 5.2 ± 0.2 nmol pNP $min^{-1}$ $\mu g^{-1}$ protein

In comparison with usual ALP inhibitors it is possible to observe that vanadate ($ED_{50} = 13\mu M$) and vanadyl ($ED_{50} = 8.7\mu M$) (Cortizo et al. 1994) specie show a stronger inhibitory effect than copper ion (this work) under similar conditions. Taking into account that the same experimental conditions to estimate ALP inhibition were used in both cases, direct comparisons with reported vanadyl complexes were performed. The methimazole copper complex ($ED_{50} = 42\mu M$) is a better inhibitor than VO-saccharide (Etcheverry et al. 2001), VO-Hesperdin (Etcheverry et al. 2008) VO-Quercetin (Ferrer et al. 2006) complexes and close to the well known hypoglycemic agent, bis(maltolato)oxovanadium (IV) BMOV ($ED_{50} = 32.1$ μM) measured at pH = 7 (Li et al. 2008).

The inhibitory effect on the activity of the enzymes that catalyze phosphoryl group transference can be attributed to a better bioavailability rather than to the increased potency at the phosphatase enzyme active sites caused by metal complexes and ligands (Peters et al. 2003). In this work it was demonstrated that the cation (in a less extent), the ligand and the complex exerted a toxic effect by blocking functional groups of enzyme. For the complex, this fact may be due to the coordination of the copper(II) cation to the sulfur moiety of the methimazole, leaving the interacting part of the ligand unmodified. The complexation improved the inhibition power of methimazole. In this context, the possibility that in solution the complex adopted a more appropriate symmetry in the interaction with the enzyme (Ferrer et al. 2005) is suggested. It can then be concluded that in addition to the antithyroid action of methimazole, this compound and its copper-complexes may provide a promising

source for research and development of potential drugs for phosphatase inhibition.

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