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Complex formation equilibria of Cu^{II} and Zn^{II} with triethylenetetramine and its mono- and di-acetyl metabolites†

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Triethylenetetramine (TETA) dihydrochloride, or trientine, is a therapeutic molecule that has long been used as a copper-chelating agent for the second-line treatment of patients with Wilson's disease. More recently, it has also been employed as an experimental therapeutic molecule in diabetes where it improves cardiac structure in patients with diabetic cardiomyopathy and left-ventricular hypertrophy. TETA is metabolized by acetylation, which leads to the formation of two main metabolites in humans and other mammals, monoacetyl-TETA (MAT) and diacetyl-TETA (DAT). These metabolites have been identified in the plasma and urine of healthy and diabetic subjects treated with TETA, and could themselves play a role in TETA-mediated copper chelation and restoration of physiological copper regulation in diabetes. In this regard, a potentiometric and spectrophotometric study of Cu^{II}-complex formation equilibria of TETA, MAT and DAT is presented here, to provide a comprehensive evaluation of the stoichiometries of the complexes formed and of their relative stability constants. A potentiometric study has also been conducted on the corresponding Zn^{II} complexes, to evaluate any possible interference with TETA-mediated Cu^{II} binding by this second physiological transition-metal ion, which is present in similar concentrations in human plasma and which also binds to TETA. An ESI-MS study of these systems has both confirmed the complex formation mechanisms established from the potentiometric and spectrophotometric results, and in addition provided direct information on the stoichiometry of the complexes formed in solution. These data when taken together show that the 1 : 1 complexes formed with Cu^{II} and Zn^{II} have different degrees of protonation. The stability of the Cu^{II} and Zn^{II} complexes with the three ligands, evaluated by the parameters pCu and pZn, decreases with the introduction of the acetyl groups. Nevertheless the stability of Cu^{II} complexes with MAT is sufficiently high to enable its participation in copper scavenging from the patient. A speciation study of the behavior of TETA and MAT with Cu^{II} in the presence of Zn^{II} at peri-physiological plasma concentrations is also presented. While Zn^{II} did not hinder copper binding, the possibility is raised that prolonged TETA treatment could possibly alter the homeostatic regulation of this essential metal ion. The lack of reliable literature stability constants concerning the Cu^{II} and Zn^{II} interaction with the major transport proteins in plasma is also briefly considered.

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Introduction

Triethylenetetramine (TETA) dihydrochloride is a therapeutic molecule mainly used in the treatment of Wilson's disease, a human disorder characterized by copper accumulation in certain organs including the liver, brain and eye.[1,2]

In 1985 the U.S. Food and Drug Administration approved TETA as an orphan drug for treatment of penicillamine-intolerant patients with Wilson's disease.

In 1993 Kodama *et al.* found a TETA metabolite in the urine when TETA was orally administered to healthy volunteers.[3] The same authors successfully identified this metabolite as N^1-acetyltriethylenetetramine (MAT).[4]

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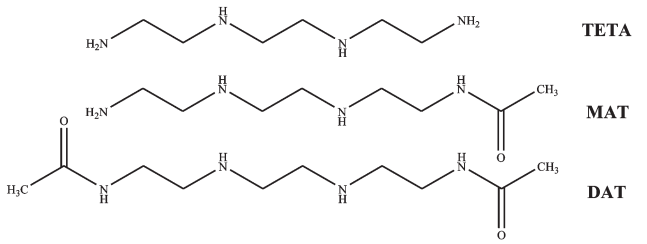

Fig. 1 Molecular structures of the three ligands studied here.

In a more recent study of Lu *et al.*,[5] besides MAT a second major metabolite of TETA was detected in human urine, N^1, N^{10}-diacetyltriethylenetetramine (DAT) (Fig. 1).

Both metabolites were verified by LC-MS with synthetic standards. The proportion of unchanged TETA excreted as a fraction of total urinary drug-derived molecules was significantly higher in healthy than in matched diabetic subjects, consistent with a higher rate of TETA metabolism in the latter. TETA-evoked increases in urinary copper excretion in non-diabetic subjects were more closely correlated with parent drug concentrations than in diabetic subjects wherein, by contrast, urinary copper was more closely associated with the sum of TETA and MAT. These findings are consistent with the hypothesis that MAT could play a significant role in the molecular mechanism by which TETA extracts Cu^{II} from the systemic compartment in diabetic subjects.

These findings seem to imply a strong interaction of MAT with Cu^{II}, contrary to that reported in the work of Kodama *et al.*,[3] wherein it is stated that "the chelating activity of acetyltrien (MAT) is much lower than that of trien (TETA)". This statement would seem to be based on an incomplete qualitative interpretation of the titration curves of TETA and MAT, both pure and in the presence of Cu^{II}, consistent with current understanding of solution equilibria studies. Given the strong biomedical relevance of the knowledge of the formation constants between copper and MAT and DAT established by these aforementioned observations, we performed a potentiometric and spectrophotometric study of their complex formation equilibria.

We furthermore carried out a study of the already known equilibria between TETA and Cu^{II} in order to provide for comparison the related stability constants measured under the same experimental conditions. In addition, the study was extended to analyze complex formation between Zn^{II} and TETA and its metabolites, to characterize any possible interference with copper chelation, since zinc is a prevalent divalent transition metal ion in human plasma and, furthermore, is known to bind to TETA.

Experimental section

Materials

TETA dihydrochloride (98%) (**1**) was purchased from Sigma-Aldrich, whereas MAT trihydrochloride (98.50%) (**2**) and DAT dihydrochloride (95.43%) (**3**) were synthesized by Carbogen AG (Hunzeschwil, Switzerland) and used without any further purification. HCl was purchased from Fluka, KCl and ZnO from Carlo Erba (Milan, Italy), and KOH and $CuCl_2$ were Sigma-Aldrich products. A previously described method was used in the preparation of 0.1 M carbonate-free KOH solution.[6] TETA dihydrochloride solution was acidified with two equivalents of HCl. Cu^{II} solution was prepared by dissolving the required amount of $CuCl_2$ in pure water to which a stoichiometric amount of HCl was added to prevent hydrolysis. This solution was standardized by volumetric EDTA titration. A Zn^{II} standard solution was prepared by dissolving in double-distilled water the required amount of ZnO to which three stoichiometric amounts of HCl were added.

Potentiometric–spectrophotometric measurements

Protonation and complex-formation equilibrium studies were carried out under the same conditions described in a previous publication.[7]

The operative ligand concentrations were 1.5×10^{-3} M for TETA, 1.6×10^{-3} M for MAT and 1.8×10^{-3} M for DAT. The studies of complex formation were performed using constant ligand concentration, and 1 : 1 and 1 : 2 metal/ligand molar ratios.

Combined potentiometric–spectrophotometric measurements were performed for Cu^{II} complexes, while only potentiometric data were collected for non-absorbing Zn^{II} complexes.

Spectra of Cu^{II} complexes with all the ligands were recorded in the 200–400 nm spectral range using a 0.2 cm path length. In the case of TETA the visible 400–800 nm range was also examined, with a 1.0 cm path length and a ligand concentration of 5.0×10^{-3} M. Protonation and complex formation data were analyzed by using the Hyperquad program.[8]

Electrospray ionization-mass spectrometry analysis of complexes

Mass spectra were recorded in positive-ion mode on a Bruker micro TOF-Q spectrometer (Bruker Daltonics, Bremen, Germany), with an electrospray ionization (ESI) source. Conditions of the experiment were similar to those published previously.[9] The capillary temperature was reduced to 170 °C in order to increase the abundance of the peaks corresponding to complexes. The samples were dissolved in water at neutral pH with addition of methanol up to 50%. This allowed us to reduce capillary temperature in order to obtain good spectra-temperature sensitive complexes. The ligand concentration was approximately 5×10^{-4} M. Complexes with $CuCl_2$ and $ZnCl_2$ solutions were prepared at least 10 min before each experiment, at a ligand to metal molar ratio of 10 : 1. The distance between isotopic peaks was taken into consideration for calculation of the overall charge of the analyzed ions. Simulated spectra were calculated by using the Compass DataAnalysis program (Bruker Daltonics). The figures for actual and simulated spectra are presented to verify the stoichiometry of complexes: similar isotopic distributions in the actual and

simulated spectra confirm the accuracy of the proposed chemical structures.

Results and discussion

Protonation constants of the pure ligands

The protonation constants of the pure ligands were determined by potentiometric measurements at 25 °C and ionic strength generated by 0.1 M KCl. The data reported in Fig. 2 were processed by using the Hyperquad program[8] and the stability constants are reported in Table 1.

The four protonation constants of TETA listed in Table 1 are comparable with the 9 sets of constants determined under the same conditions, 25 °C and 0.1 M ionic strength, and reported in the literature.[10] The values log K_1 = 9.8(2), log K_2 = 9.1(1), log K_3 = 6.7(1) and log K_4 = 3.4(1) are the mean values of the 9 protonation constants reported in the literature and the numbers in parentheses are the relative standard deviations. The variability of data in different literature reports depends on the variable performance by different groups; the more scattered values of log K_1 probably reflect the high pH values employed in their determination. The best way to determine accurate protonation constants at high pH values is to use spectrophotometric approaches;[11] however, since free TETA does not absorb in the entire UV-Vis spectral range, use of this methodology to study its behaviour is precluded.

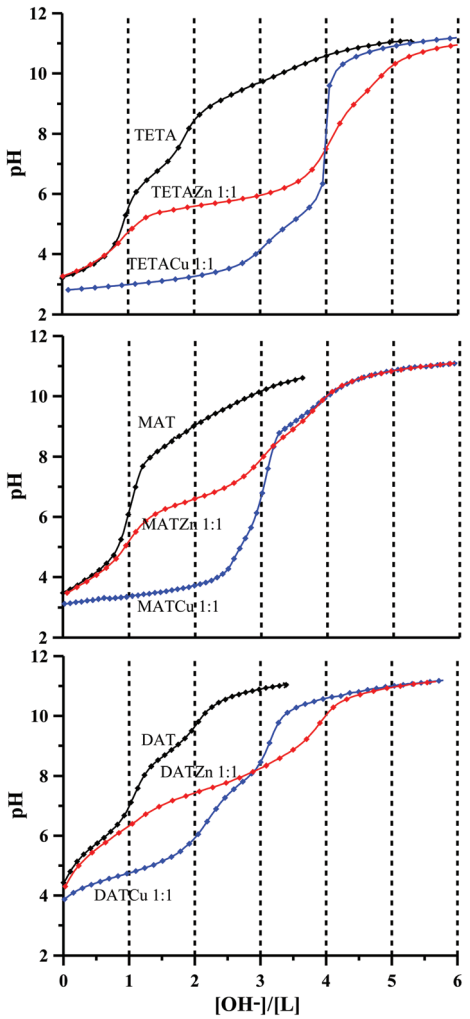

Fig. 2 Potentiometric titrations of the pure ligands (black), of 1 : 1 Cu–ligand systems (blue), and of 1 : 1 Zn–ligand systems (red). The experimental curves are reported as points, and, as continuous lines, those calculated using the protonation and complex formation constants in Table 1.

Table 1 Protonation (log K) and complex formation (log β) constants determined at 25 °C and 0.1 M ionic strength in the pH range

	Model			Ligand		
	M	L	H	TETA	MAT	DAT
	0	1	1	9.79(5)	9.71(6)	8.61(1)
	0	1	2	9.11(4)	8.27(1)	5.73(2)
	0	1	3	6.68(2)	4.17(1)	—
	0	1	4	3.28(2)	—	—
Cu	1	1	1	23.4(1)	—	—
	1	1	0	20.3(1)	14.9(1)	7.93(4)
	1	1	−1	—	5.7(2)	0.71(4)
	1	1	−2	—	—	−9.57(6)
	pCu			17.1	12.6	8.0
Zn	1	1	1	18.06(6)	14.40(5)	—
	1	1	0	12.24(3)	7.88(3)	4.50(5)
	1	1	−1	2.90(6)	−1.15(4)	−2.98(4)
	1	1	−2	—	—	−11.49(4)
	pZn			8.4	6.2	6.0

The question of the attribution of the four protonation constants to individual nitrogen atoms in tetra-amines such as TETA and related compounds has been thoroughly examined by Barbucci *et al.*,[12] who have stated that the difference between the first (log K_1) and second (log K_2) protonation constants in TETA may be accounted for by statistical effects connected with the protonation of two equivalent groups. In such a case the ratio between the two protonation constants is 4, corresponding to a difference of 0.6 between the log K values. This finding indicates that the positive charge on the molecule due to the first protonation event does not affect the basicity of the nitrogen atom protonated in the second step. Such behaviour can be observed when the two nitrogen atoms are sufficiently far apart, and supports the conclusion that the first two protons are bound by the two terminal primary-amine nitrogen atoms.[12] The constants log K_3 and log K_4 relate to the protonation of the two inner nitrogen atoms. As a consequence, the repulsion of the one or two vicinal protonated secondary-amine nitrogen atoms causes the marked decrease in log K_3 (~2.4 units) and log K_4 (~3.4 units).

In the case of MAT, the initial protonation of the terminal primary-amine nitrogen atom is similar to that in TETA; in this case also, the distance between the two peripheral nitrogen atoms prevents any influence of the acetyl group. Both the shorter distance from the positively-charged nitrogen atom and the proximity to the acetylated nitrogen atom interact to lower the log K_2 value (~1.4 units) relative to the protonation of the inner nitrogen atom closer to the substituted nitrogen; the basicity of the remaining inner nitrogen (log K_4) is further

strongly decreased by the two vicinal charged nitrogen atoms (~4.1 units).

The di-acetylated molecule, DAT, has only the two inner nitrogen atoms that are subject to protonation. The log K_1 value in DAT is similar to the log K_2 value in MAT, but slightly higher since the initial proton is entering into an uncharged molecule. The log K_2 value is also higher than the corresponding log K_3 in MAT, since it is acted on by only one vicinal charge. The protonation constants log K_1 = 8.79 and log K_2 = 5.79, reported previously for MAT,[13] are consistent with those presented in Table 1; the modest difference in values derives from different temperatures (20 °C *vs.* 25 °C) and different electrode calibration methods.

Cu^{II} complex formation equilibria

The availability of pure TETA allowed us to validate the experimental procedures before embarking on systematic studies of systems containing the two metabolites. These complex formation equilibria have been studied potentiometrically and by UV-Vis spectrophotometry. The formation of copper complexes is accompanied by the appearance of two bands, the first in the UV region centred at ~250 nm with an absorptivity of ~2400 M^{-1} cm^{-1}, and the second in the visible region at ~580 nm with a lower absorptivity of ~60 M^{-1} cm^{-1}.

The potentiometric titration curves are presented in Fig. 2, the UV spectra collected during the titration in Fig. 3A, and the spectra collected in the visible range at five-fold higher concentrations of ligand and Cu^{II} in Fig. 3B. From the spectra in Fig. 3A it can be seen that the complex had already partially formed at the starting pH of the titration. For this reason, the set of visible spectra shown in Fig. 3B were collected by titrating the Cu–TETA mixture with both KOH (0.1 M) and HCl (0.1 M).

These data were analysed by application of the Hyperquad program.[8] The results show the formation of a 1 : 1 complex CuLH only, at low pH, which transforms into CuL at about pH 3. The relative stability constants are reported in Table 1.

An analysis of literature data shows that at least three different models have been proposed. A first one, proposed by Tabushi *et al.* and by Hulanicki *et al.*, takes into account the formation of CuL alone, characterized by a log β_{11} ranging from 20.1 to 20.4.[14] A second model, proposed by three different research groups, considers the formation of three differently protonated 1 : 1 complexes from CuLH to $CuLH_{-1}$.[15]

The third model proposed by Laurie and Sarkar considers, besides the variously protonated 1 : 1 complexes, also the existence of the 1 : 2 complexes CuL_2H_4, CuL_2H_3 and CuL_2.[16] The 1 : 2 complexes in the latter model of Laurie and Sarkar contribute less than 20% of the total copper complexation until pH 6, at TETA 10^{-3} M and Cu^{II} 10^{-4} M; at physiological pH 7.4 the prevailing species is always the CuL complex, and the presence of CuL_2H_3 falls to less than 5%. All the speciation plots calculated according to the different models in the literature show the CuL complex to be the prevailing species at pH 7.4 with the $CuLH_{-1}$ species appearing only at pH values above 9–10. In the literature, the calculated pCu values range from 16.6 to 19.9. Our result, 17.1, agrees well with these.

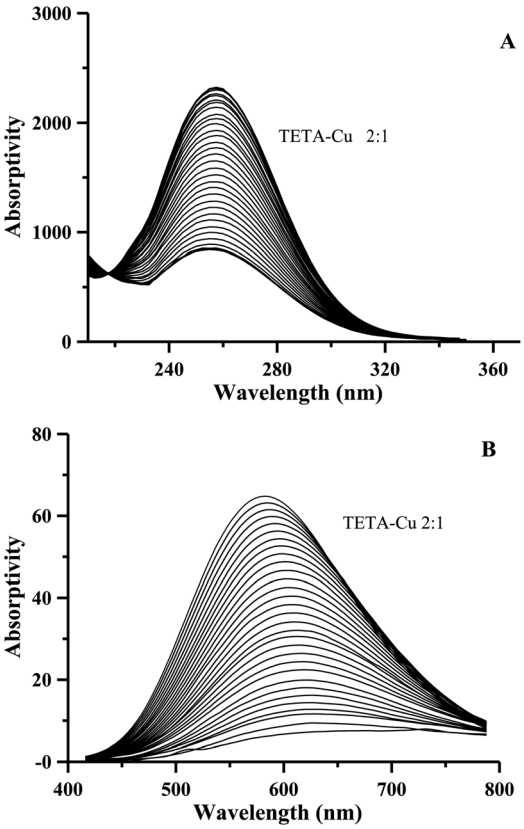

Fig. 3 (A) UV spectra collected during potentiometric titration of the system TETA:Cu^{II} at a 2 : 1 molar ratio from pH 2.9 to pH 10.1 (absorptivity increases with pH), C_{TETA} 1.5 × 10^{-3} M, using a 0.2 cm optical path length; (B) visible spectra of the system TETA:Cu^{II} at a 2 : 1 molar ratio from pH 1 to pH 10.2 (absorptivity increases with pH), C_{TETA} 5 × 10^{-3} M, using a 1.0 cm optical path length.

Equivalent potentiometric and UV spectrophotometric studies were carried out on the complex formation equilibria of MAT and DAT with Cu^{II}. The titration curves are shown in Fig. 2 and the UV spectra collected in the course of the titrations are shown in Fig. 4 and 5, respectively. Potentiometric and spectrophotometric data were elaborated using the Hyperquad program and the complex formation models together with their relative stability constants are presented in Table 1.

MAT forms a CuL complex that loses one proton, presumably from a coordinated water molecule, at pH ~ 9.2. The formation of the MAT hydroxo-complex is accompanied by a shift of the peak at 269 nm to lower wavelengths (~245 nm), with a clear isosbestic point at ~265 nm (Fig. 4). DAT forms a less stable CuL complex that loses two protons from coordinated water molecules, the first one at pH ~ 7.2, and the second at pH ~ 10.3. Spectral behaviour similar to that of MAT was also observed in the case of the di-acetyl derivative, DAT (Fig. 5).

A first comparison of the chelating properties of TETA metabolites with respect to those of the parent molecule can be made by observing the speciation plots reported in Fig. 6. A

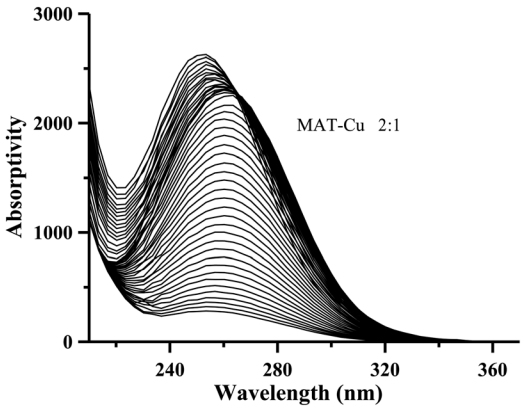

Fig. 4 UV spectra collected during potentiometric titration of the system MAT: Cu^{II} at a 2 : 1 molar ratio from pH 3.3 to pH 10.0 (absorptivity increases with pH), C_{MAT} 1.6×10^{-3} M, using a 0.2 cm optical path length.

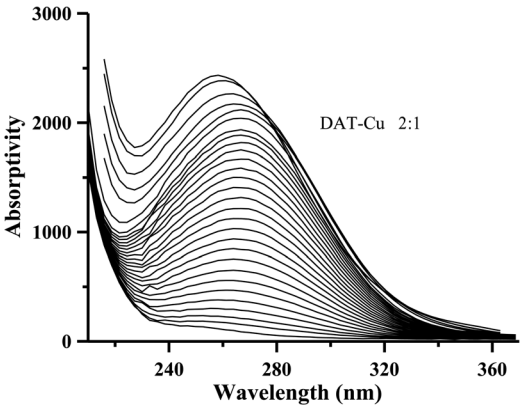

Fig. 5 UV spectra collected during potentiometric titration of the system DAT: Cu^{II} at a 2 : 1 molar ratio from pH 4.0 to pH 10.1 (absorptivity increases with pH), C_{DAT} 1.8×10^{-3} M, using a 0.2 cm optical path length.

decrease of stability is clear from the fact that the complex formation takes place at increasing pH values passing from TETA, to MAT and DAT. A second observation that can be made is the formation of hydroxo-complexes with MAT and DAT, due to the deprotonation of coordinated water molecules. These deprotonation events, which take place at pH ~ 9.2 in MAT and at pH ~ 7.2 and ~10.3 in DAT, probably play a role in stabilizing the Cu^{II}–MAT and Cu^{II}–DAT complexes of decreasing stability. A more detailed examination has been presented in the Discussion section, below.

Zn^{II} complex formation constants

The formation of zinc complexes has been studied by potentiometry alone. The titration curves, presented in Fig. 2 together with those for copper, give some direct insight into the formation of Zn^{II} complexes: ZnL complexes are formed, as in the case of copper, with all three ligands: all are of remarkably lower stability than the corresponding copper complexes. The models and the stability constants are reported in Table 1. As can be seen both from the titration curves and from the speciation plots (Fig. 6) the zinc coordination takes place at pH values about 2.4 pH units higher than those of copper complexes with the corresponding ligand.

ESI-MS characterization of Cu^{II} and Zn^{II} complexes

TETA. The ESI-MS spectrum of TETA complexes with Cu^{II} is shown in Fig. 7a. The signals at *m*/*z* 208.075 and 244.051 represent mononuclear complexes with stoichiometry $[CuT]^+$ and $[CuT(H_2O)_2]^+$ respectively.

The signal at *m*/*z* 178.042 corresponds to a complex of molecular formula $C_5H_{13}N_3Cu$. Formation of this ion can be explained by the α-cleavage reaction of the ligand in $[CuT]^+$. No peaks corresponding to $[CuT(H_2O)]^+$ were observed, consistent with instability of the putative $[CuT(H_2O)]^+$ complex (Fig. 7b). Further data consistent with this hypothesis were generated by the MS/MS experiment shown in Fig. 7c. Collision induced dissociation (CID) of $[CuT(H_2O)_2]^+$ results in the loss of two water molecules to give $[CuT]^+$. Fig. 8a represents TETA complexes with Zn^{II} ions. The complexes $[ZnT]^+$, $[ZnT(H_2O)]^+$ and $[ZnT(H_2O)_2]^+$ are represented by the peaks at *m*/*z* 209.074, 227.084, and 245.050 respectively. CID of $[ZnT]^+$ leads to the formation of two complexes (Fig. 8b). The peak at *m*/*z* 190.032 corresponds to a molecular formula of $C_6H_{11}N_3Zn$, while that at *m*/*z* 162.002 corresponds to $C_4H_7N_3Zn$. In contrast to the corresponding Cu^{II} complexes, MS/MS fragmentation of the complex $[ZnT(H_2O)_2]^+$ leads to step-by-step cleavage of water molecules (Fig. 8c). These findings are consistent with the hypothesis that monohydrated complexes of TETA with Zn^{II} are stable.

MAT. The stoichiometry of MAT–Cu^{II} complexes is the same as that observed for TETA–Cu^{II} complexes. The $[CuM]^+$ and $[CuM(H_2O)_2]^+$ ions are formed, as represented by the peaks at *m*/*z* 250.085 and 286.061 (Fig. 9a), whereas no ions corresponding to $[CuM(H_2O)]^+$ were observed.

The $[CuM(H_2O)_2]^+$ ion was selected for a further MS/MS experiment (Fig. 9b). The carbon–carbon bond fragmentation of the ligand, resulting from α-cleavage of $AcNHCH_2$–CH_2, is represented by the peak at *m*/*z* 220.051. Additionally, the peak at *m*/*z* 178.040 was present, consistent with the previous result from the TETA–Cu^{II} sample, and corresponds to α-cleavage of the ligand–copper complex with the carbon–carbon bond (in NH_2CH_2–CH_2).

Fig. 10a shows the ESI-MS spectrum of the $[ZnM(H_2O)_2]^+$ complex with a peak at *m*/*z* 287.059. The MS/MS experiment leads to cleavage of water molecules with formation of $[ZnM]^+$ as the remaining zinc complex, represented by the peak at *m*/*z* 251.084 (Fig. 10b).

DAT. The ESI-MS spectrum of DAT–Cu^{II} (Fig. 11a) shows the formation of the complexes $[CuD]^+$ (*m*/*z* 292.093) and $[CuD(H_2O)_2]^+$ (*m*/*z* 328.071).

No peaks corresponding to DAT–copper monohydrate complexes were observed. MS/MS of the *m*/*z* 292.093 ion (Fig. 11b) led to formation of the $[C_7H_{15}CuN_3O]^+$ ion, which was also observed in Fig. 9b, and corresponds to the copper complex after α-cleavage of the carbon–carbon bond in NH_2CH_2–CH_2.

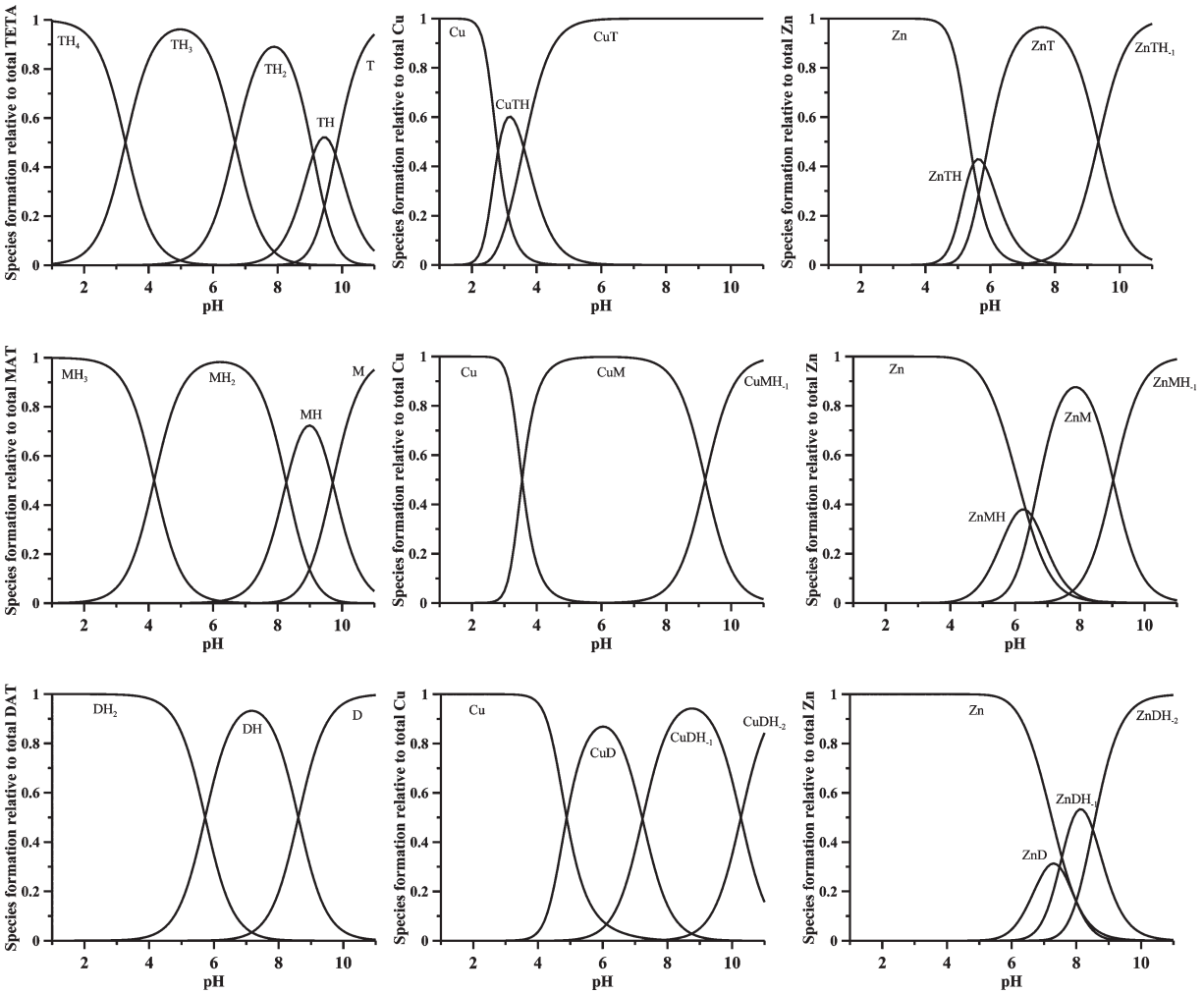

Fig. 6 Speciation plots relative to the protonation of the three pure ligands (left column), to copper complex formation (center), and to zinc complex formation (right). The letters T, M, and D on the plots stand for TETA, MAT, and DAT and charges have been omitted for simplicity. The speciation plots have been calculated using the protonation and stability constants in Table 1. Ligand concentration was always 1×10^{-3} M and a 1 : 1 metal:L ratio has been used for metal complex formation.

CID performed on the ion of *m*/*z* 328.071 (Fig. 11c) instead led to cleavage of two water molecules and α-cleavage of the ligand.

The signal at *m*/*z* 329.065 in Fig. 12a is indicative of the presence of the $[ZnD(H_2O)_2]^+$ complex. CID fragmentation of the $[ZnD(H_2O)_2]^+$ ion caused formation of the $[ZnD]^+$ ion (Fig. 12b). The comparisons between the observed and calculated peaks relative to the presented data are reported in the ESI,† and are in perfect agreement.

Discussion

All the experimental techniques employed, namely potentiometry, UV-Vis spectrophotometry and ESI-MS, have shed light on the formation of 1 : 1 Cu^{II} and Zn^{II} complexes with all three ligands, with different degrees of protonation. The introduction of each new acetyl group into the original scaffold of TETA leads to a variation of the protonation behaviour of the ligands, with increases in the corresponding protonation constants for the three ligands, and decreases of the complex formation constants. The comparison of the chelating properties of TETA metabolites with respect to those of the parent molecule requires understanding of some preliminary considerations: the real effective binding capacity of different ligands depends on a variety of factors, the main ones being the metal-proton competition for the same basic sites on the ligand, and the stoichiometry of the formed complexes.

Different parameters have been proposed in the literature to evaluate this effective binding capacity;[17] the parameter $pM = -\log_{10}(Mf)$ (calculated at $[M]_T = 10^{-6}$ M, $[L]_T = 10^{-5}$ M and pH = 7.4), proposed by Harris *et al.*,[18] gives a direct and clear picture of the strength of a chelator toward a target metal ion. Thus comparison of the binding capacity of TETA, MAT and DAT toward copper ions can be made on the basis of pCu values, thereby taking into consideration the different protonation and formation mechanisms of the respective complexes

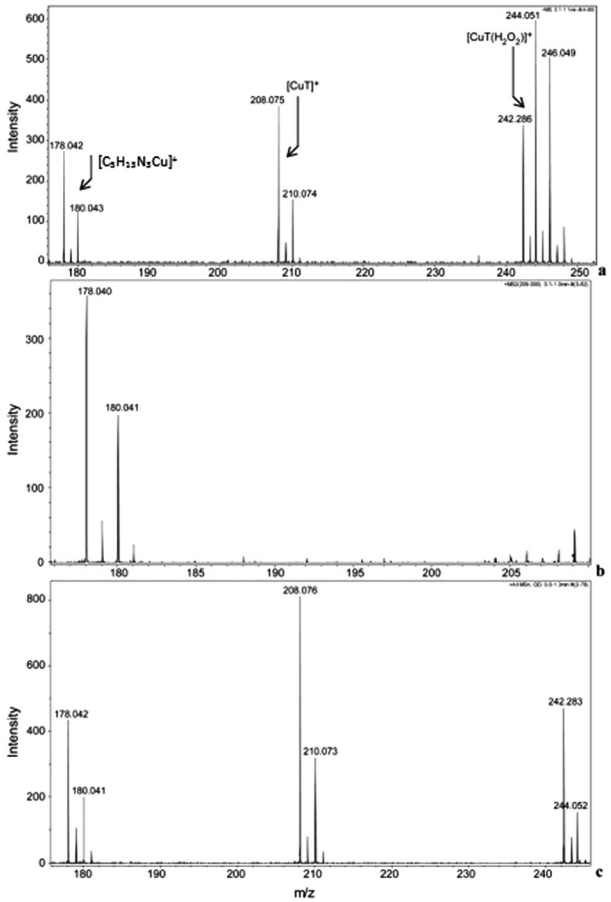

Fig. 7 (a) ESI-MS spectrum of TETA–Cu^{II} complexes; (b) ESI-MS/MS spectrum of $[CuT]^+$; (c) ESI-MS/MS spectrum of $[CuT(H_2O)_2]^+$.

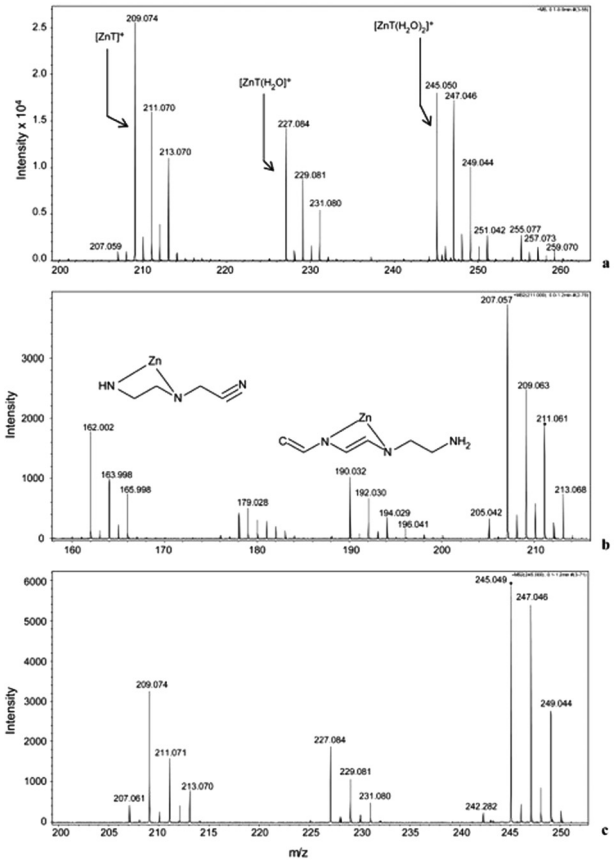

Fig. 8 (a) ESI-MS spectrum of TETA–Zn^{II} complexes; (b) CID of $[ZnT]^+$; (c) MS/MS fragmentation of $[ZnT(H_2O)_2]^+$.

under consideration. The pCu values for the three molecules have been reported in Table 1: they show a marked decrease, from 17.1 for TETA to 12.6 for MAT to 8.0 for DAT, of about 4.5 pCu units for the addition of each acetyl group. The 17.1 value describes TETA as an extremely strong chelator for copper, the 12.6 value for MAT is still good and characterizes it as a strong chelator, while the value of 8 for DAT implies that this ligand is a weak chelator, leaving 1% of copper free (not complexed), in the conditions used for pCu calculation. A similar decreasing trend with the introduction of acetyl groups is shown by the pZn values, but in this case the strength of all three ligands for zinc chelation is extremely low.

In spite of the lower stability constants for Zn^{II} of TETA and its derivatives, the relatively high zinc concentration in human plasma of this metal ion ($\sim 1 \times 10^{-5}$ to 1.5×10^{-5} M) indicates that it might produce some interference with copper chelation. To quantitatively evaluate the effects of physiological Zn^{II} on Cu^{II} chelation, in this section we now present the speciation of copper and zinc ions with the two strongest ligands TETA and MAT. Some preliminary review of the pharmacokinetic behaviour of TETA is necessary in order to derive appropriate concentrations of ligands to use in our speciation study. Most oral TETA is not absorbed, but excreted unchanged in the faeces.[19]

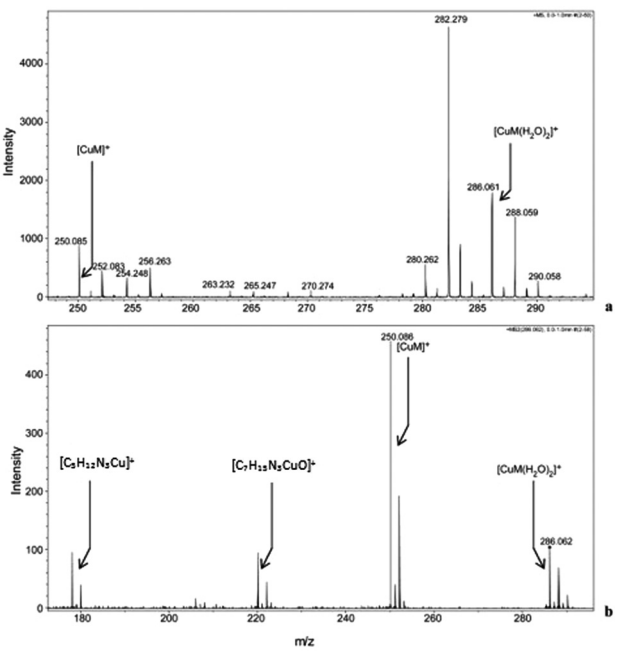

Fig. 9 (a) ESI-MS spectrum of MAT–Cu^{II} complexes; (b) CID fragmentation of $[CuM(H_2O)_2]^+$.

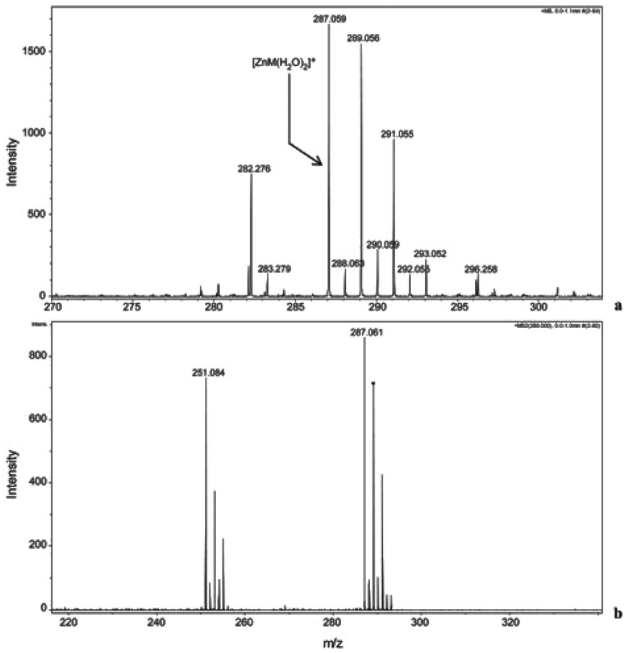

Fig. 10 (a) ESI-MS spectrum of MAT–Zn^{II} complexes; (b) MS/MS analysis of $[ZnM(H_2O)_2]$.

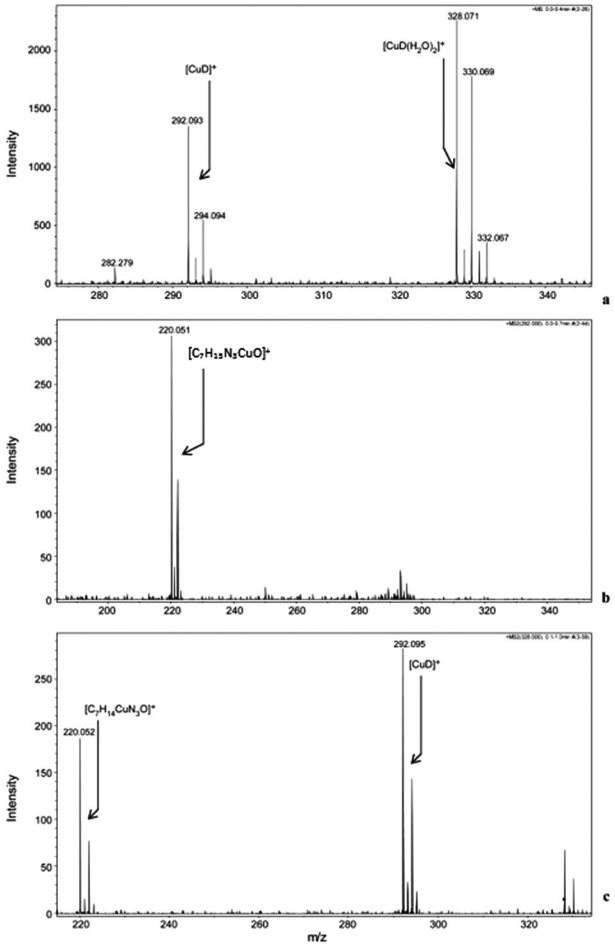

Fig. 11 (a) ESI-MS spectrum of DAT–Cu^{II} complexes; (b) MS/MS of the *m*/*z* 292.093 ion; (c) CID of the *m*/*z* 328.071 ion.

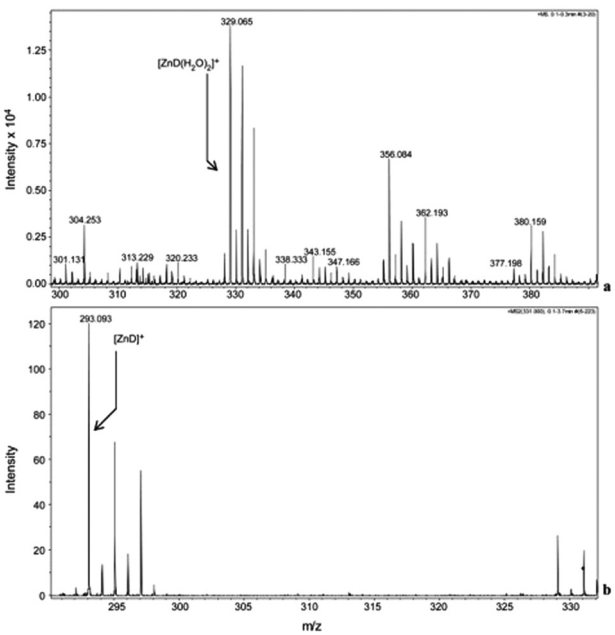

Fig. 12 (a) ESI-MS spectrum of the DAT–Zn^{II} complex; (b) MS/MS fragmentation of $[ZnD(H_2O)_2]^+$.

In studies on healthy humans it was found that the 5 to 18% of TETA that is systemically absorbed is extensively metabolized, and a urinary recovery of about 1% has been determined, whereas that of MAT was about 8%.[3,4] A paper by Lu *et al.* reported a urinary recovery between 0.03 and 13.4% of administered dose in healthy volunteers and from 3.7 to 14.6% in diabetic patients.[5] In the same study, it was also reported that unchanged TETA accounted for 22.8% ± 0.6 of total drug excreted by healthy volunteers and 7.1% ± 0.8 by diabetic patients, and that maximum concentrations of TETA and its metabolites, MAT and DAT, in the plasma of healthy subjects were 5.4 ± 2.3, 4.0 ± 1.6 and 1.1 ± 0.6 μM respectively, and in that of diabetic subjects were 6.3 ± 4.2, 10.5 ± 5.3 and 2.6 ± 1.6 μM respectively. A study using ^{13}C TETA showed that only 6 to 18% of orally administered drug was systemically absorbed.[19]

A further study has reported a bioavailability of TETA of about 13.8%.[21] Based on the above reports, a blood concentration of 4.5×10^{-5} M can be estimated for a single dose of 600 mg of orally-administered TETA, assuming 10% absorption of the drug, totally circulating in 6 L of plasma.

In the following speciation analysis 4.5×10^{-5} M was assumed to be the maximum TETA concentration before any metabolic transformation (case 1), while the values reported by Lu *et al.*[5] for healthy and diabetic subjects have been assumed as representative of two different situations, after the metabolic processing of the parent drug (cases 2 and 3 respectively). The total zinc concentration circulating in plasma has been assumed in all the three cases as 1.5×10^{-5} M.[22,23] In the case of copper, according to Linder *et al.*,[24] an amount of about 6 mg circulates in plasma differently bound to various

Table 2 Starting concentrations of TETA and MAT (T_T and M_T), and concentrations of free copper (Cu_F), free zinc (Zn_F) and of all formed complexes at equilibrium (T and M stand for TETA and MAT). Total copper concentrations were (a) 1.5×10^{-5} M and (b) 5×10^{-6} M. Total zinc was always 1.5×10^{-5} M. All the concentrations in this table are multiplied by 10^6. The HYSS program[20] has been used for speciation calculation, using protonation and complex formation constants in Table 1

	Case 1		Case 2		Case 3	
Species	a	b	a	b	a	b
T_T	45	45	5.4	5.4	6.3	6.3
M_T	—	—	4.0	4.0	10.5	10.5
Cu_F	7.6×10^{-11}	1.5×10^{-11}	5.6	5.4×10^{-7}	1.8×10^{-5}	2.0×10^{-7}
Zn_F	8.4×10^{-3}	5.1×10^{-3}	15	12.7	14.2	9.8
CuT	15.0	5.0	5.40	4.46	6.26	4.38
CuM	—	—	3.94	0.53	8.61	0.61
$CuMH_{-1}$	—	—	0.06	0.01	0.13	0.01
ZnTH	0.38	0.38	—	0.02	—	0.05
ZnT	14.4	14.4	—	0.90	0.04	1.85
$ZnTH_{-1}$	0.17	0.17	—	0.01	—	0.02
ZnMH	—	—	—	0.16	0.08	0.38
ZnM	—	—	—	1.18	0.64	2.85
$ZnMH_{-1}$	—	—	—	0.03	0.01	0.07

endogenous transport proteins, corresponding to a 1.5×10^{-5} M total copper concentration.

Each of the three cases was further divided into two subcases, a and b. In the first the copper concentration has been assumed to be 1.5×10^{-5} M, in the second 5×10^{-6} M, considering only the non-caeruloplasmin bound exchangeable copper in plasma. In Table 2 are reported the starting concentrations of TETA and DAT, and the concentrations of free copper, free zinc and of all formed complexes at equilibrium. For the speciation calculation we used the HYSS program,[20] and the protonation and complex formation constants reported in Table 1. When an excess of TETA with respect to copper is present in blood (case 1), copper is completely chelated as CuT in both a and b subcases. The ligand not complexed to copper binds zinc into the three differently-protonated zinc complexes.

In case 2a, in which copper is in excess with respect to the sum of MAT and DAT, the two ligands chelate copper according to their respective concentrations, leaving any excess copper unchelated; in this case, zinc remains totally unchelated. In case 2b all copper is completely complexed by the two ligands in amounts dependent on the relative concentrations of TETA and MAT, and on their stability constants. The excess of chelator molecules with respect to copper is utilized in zinc complexation. A similar situation is observable in cases 3a and b.

The remarks which can be inferred from these simulations are that zinc does not interfere with copper chelation in any of the examined situations, but whenever the ligands are in excess with respect to chelatable copper, zinc is complexed by the excess of ligand. Thus, depending on the relative concentrations of metal ions, TETA and its metabolites, the possibility exists that chronic TETA treatment could significantly perturb zinc homeostasis. Cooper *et al.* have undertaken a detailed study of the effects of TETA treatment on zinc homeostasis in two clinical trials. In one, they performed a balance study in 20 type-2 diabetic patients and 20 matched non-diabetic controls at a clinically-effective dosage.[23] TETA, administered as trientine, elevated urinary zinc excretion in both non-diabetic volunteers and patients: it also increased zinc balance in the volunteers but this effect was less-marked in diabetic patients.[25] In this study, increased zinc uptake was balanced by increased urinary zinc excretion. These effects are consistent with previous observations in rodents, where high doses of TETA were shown to increase systemic zinc values.

Cooper *et al*. have also performed a 12-month clinical trial in which they measured the effects of TETA on left-ventricular mass in type 2 diabetic patients with left-ventricular hypertrophy, where they found that serum zinc concentrations were unaffected over 12-months treatment.[26] Thus, the increased zinc uptake caused by TETA treatment was probably balanced by increased urinary zinc excretion.

The speciation results presented have been derived by a simple model which has not taken into account the complexation of both metal ions by transport proteins in plasma, such as caeruloplasmin, albumin, transcuprein, *etc.*,[19] but the poor literature reference values (few and mutually inconsistent data) prevented their use in this calculation. Furthermore in our opinion in such situations the distribution of the metal ions among different species is not only dictated by the thermodynamic properties of the systems, but by the kinetic ones too.[27]

Conclusions

The present study has allowed the evaluation, by application of complementary experimental techniques, of the complexes formed by TETA and by its two acetyl derivatives with copper and zinc. In particular the potentiometric and spectrophotometric results have allowed the thorough definition of the copper complex formation equilibria, and the potentiometric data alone to define zinc complexation equilibria. The ESI-MS results have supported the 1 : 1 stoichiometric models determined by equilibrium analysis; they give furthermore evidence of the fact that copper can be complexed by the three ligands

without any coordinating water molecule, or with two coordinating water molecules. ESI-MS results indicate that zinc behaves similarly to copper in this regard, but that its complexes can also be stabilized by a single coordinated water molecule in a penta-coordinated conformation.

The speciation study has given evidence that MAT could play a strong role in copper complexation, supporting the chelating action of the parent TETA. The same study has also pointed out the necessity to control the homeostatic equilibria of endogenous essential transition-metal ions, such as Zn^{II} which could be perturbed by such chelation therapies.

A final point to be stressed is the need for further experimental analysis, using both thermodynamic and kinetic approaches, of the interaction of metal chelators with the target toxic metal ions in the presence of the endogenous macromolecular ligands.

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