

Stability of Benzotriazole Derivatives with Free Cu, Zn, Co and Metal-Containing Enzymes: Binding and Interaction of Methylbenzotriazoles with Superoxide Dismutase and Vitamin B₁₂

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Abstract: Benzotriazole derivatives form very strong bonds with transition metals, and are the most widely used type of industrial corrosion inhibitor. Some benzotriazole derivatives have been implicated as hormone regulators which also carry the ability to induce uncoupling responses or otherwise inhibit respiration processes in some microorganisms. However, the mechanisms associated with benzotriazole toxicity and inhibition are unknown. Using Differential Pulse Polarography, the stability constants of commercially significant corrosion inhibitors, 4-and 5-methylbenzotriazole, coordinated with free Cu (II) and Co (III), were determined to be 10^{15} and 10^8 , respectively. Polarographic analyses were extended to confirm that methylbenzotriazole also binds the copper center(s) in the ubiquitous enzyme superoxide dismutase, and the Corrin site in the coenzyme cobalamin (vitamin B₁₂). These results suggest that the metal-chelating ability of this unique class of compounds may confer inhibition to certain enzyme systems.

1. Introduction

Benzotriazoles (BTs) and its derivatives are widely used in industry, agriculture, and in medicinal chemistry. They have been used as corrosion inhibitors, deicing/anti-icing fluids, radio protectors, and rubber and chemical fiber [1- 6]. They have been also used as UV stabilizers in plastics, and polymer stabilizers in photographic industry [7]. Beside the enormous industrial use, BTs and its derivatives demonstrated many biological activities, such as, antibacterial, antifungal, antineoplastic, anti-inflammatory, analgesic, antimalarial and antiviral activity [8-12].

Apart from industrial and biological importance, acute and chronic toxic responses to BT and its derivatives have been observed in several fish species, zooplankton, and bacteria [1,13-16]. Previous study indicated that BT and methyl-benzotriazoles (MeBT) are toxic to the luminescent bacteria used in the Microtox® assay [14-15]. Acute toxic response to benzotriazole and its commercially significant derivatives have been observed in several fish species, zooplankton, and bacteria at concentration as low as 6 mg/L [1,13,15]. Same response of pure bacterial cultures (Microtox®) to unmethylated benzotriazole and 4- methylbenzotriazole was observed, whereas 5-methylbenzotriazole was more toxic [1]. The mechanism of toxicity however, remains unknown.

Some benzotriazole derivatives have been implicated to inhibit metal-containing oxido/reductase enzymes that are critical to respiration, or these involved with the scavenging of toxic respiration intermediates. O'Brien was the first to isolate a significant benzotriazole toxicity mechanism to pure



culture of *Pseudomonas aeruginosa* bacterial cells that was associated with respiratory chain activity: uncoupling oxidative phosphorylation from bacterial respiration [17]. Author suggested that benzotriazole toxicity may be associated with the complexation of membrane bound enzymes involved in the respiratory process. The results were with agreement with De Wever and Verachtert who also suggested that the toxicity of 2-mercaptobenzothiazole (MBT) to bacteria and other organisms is due to the complexation between metal-containing, membrane bound enzymes and benzothiazole [18]. Benzotriazoles are theorized to be successful corrosion inhibitors because of their ability to form stable complexes with different yellow metals, especially copper. Their metal chelating properties may be directed towards metals that serve as critical oxidation/reduction sites in some enzymes. Furthermore, the methyl-benzotriazole binding may significantly change the electron transport abilities such that an enzyme's catalytic properties are adversely affected.

Superoxide dismutase enzyme (SOD) and Vitamin B₁₂ are necessary for cells in nearly all living cells. Superoxide Dismutase is found almost in all eukaryotic cells and in many bacteria and forms a crucial part of the cellular antioxidant defense mechanism in many different types of cells. Several research reports suggest that some benzotriazole derivatives form stable, nontoxic, low molecular weight complexes of copper that mimic SOD activity, and have been proposed as substituted for SOD in clinical application. Skorda and coworkers reported that certain copper-methylbenzotriazole complexes can serve as good functional models for the active copper site of Cu-Zn superoxide dismutase [19]. Vitamin B₁₂ (Cobalamin) is a coenzyme that is necessary for red blood cells synthesis, maintenance of the nervous system, and growth and development in children. It is unique among all vitamins in that it contains not only a complex organic molecule, but one of the few enzymes to use the essential trace element, cobalt. Vitamin B₁₂ as usually isolated is called cynocobalmin because it contains a cyano group attached to the cobalt in the sixth coordination position. A critical coordination position of cobalt in vitamin B₁₂ is filled by dimethylbenzimidazole ribonucleotide, bound covalently by its 3'-phosphate group to one of the side chains of the corrin ring through aminoisopropanol.

In this study, the interaction of MeBT with the copper centers of superoxide dismutase enzyme (SOD), and the cobalt center of vitamin B₁₂ were characterized. Characterization was done to provide a better understanding for their interactions with methylbenzotriazole (MeBT), and determine if there is potential for the subsequent inhibition of their function. Determination of stability constants of MeBT interacted with free copper and cobalt ions was essential for this characterization. Differential Pulse Polarography (DPP) were used for the determination of consecutive and overall stability constants of metal complexes that can be reversibly reduced. Such an approach was applied to estimate the strength of metal-benzotriazole interactions, as well as for interactions with metal incorporated in the active sites of common (co)enzymes.

2. Differential Pulse Polarography and Stability Theory

The current that flows during a single life time of a dropping mercury electrode (DME) is held at a given potential in a polarography and is described by the Ilkovic Equation:

$$I_d = 708nD_o^{1/2}C_o^*m^{2/3}t^{1/6} \quad (1)$$

In which,

I_d = diffusion current (A)

n = number of electrons transferred/molecule,

m = mercury flow rate (mg/s).

t = Sampling interval(s).

D_o = diffusion coefficient (cm²/sec)

C_o^* = Concentration of oxidized species in bulk solution (mol/cm³)

The overall electrode reaction can be generally represented by:



Where, M^{x+} represent some metal ion, L^{y-} a ligand, and $ML^{(x+y)}$ a complex formed.

In their classic report, Deford and Hume (1951) defined a series of functions, where the change in reduction potential of a target ion, measured with a polarograph, can be directly related to its complexation stability by the progressive addition of a known ligand [20].

$$F_0(x) = \sum \beta_n C_L^n = \text{anti log} \left[0.434 \frac{nF}{RT} \Delta E_p \right] + \text{Log} \frac{(I_p)_s}{(I_p)_c} \tag{3}$$

In which,

β_n = overall stability constant of the n^{th} complex,

C_L = ligand concentration assuming $C_L \gg C_M$,

$(I_p)_s$ = diffusion current constant of free metals.

$(I_p)_c$ = diffusion current constant of complexed metal.

The shift in DPP potential on the addition of complexing ligand.

$$\Delta E_p = (E_p)_s - (E_p)_c \tag{4}$$

Related to Heath and Heftner and under most of the common testing conditions, $(I_p)_s$ for most inorganic species is equal to $(I_p)_c$ [21]. For this reason, equation (3) condenses to the following equation:

$$F_0(x) = \sum \beta_n C_L^n = \text{anti log} \left[0.434 \frac{nF}{RT} \Delta E_p \right] \tag{5}$$

Stability constants were obtained as follows

$$F_0(x) = \sum \beta_n C_L^n = \beta_0 + \beta_1 C_L + \beta_2 C_L^2 + \dots + \beta_n C_L^n \tag{6}$$

Where β_0 has a value of unity. The function $F_n(X)$ is introduced as follow

$$F_n(X) = \frac{F_{n-1}(X) - \beta_{n-1}}{C_L} \tag{7}$$

In order to obtain β_1, \dots, β_n , a widely-accepted graphical extrapolation method derived by Laden (1941) was applied in this research [22]. Sequential plots of $(F_n(X) \text{ vs. } C_L)$ yielded a family of curves, through which coordination chemistry between metals and benzotriazoles was determined

An independent voltammetric approach was used to verify the maximum coordination number, and magnitude of stability constants determined by DeFord-Hume regression. The maximum coordination number was determined using the Lingane method reported by Crow (1969) by plotting of $\Delta E_{1/2}$ Vs. $\text{Log } C_x$ relationship, using the Lingane equation [23]:

$$(E_p)_s = (E_p)_c - \frac{0.0591}{n} \text{Log} \beta_{mxj} - j \frac{0.0591}{n} \text{Log} C_x \tag{8}$$

β_{mxj} (the overall stability constant), and j (maximum number of ligands coordinated with a metal through a reversible series of complexing interactions), were determined for Cu (II), Co (III) and Zn (II) interactions with benzotriazoles.

This study reported the development and use of polarographic methods for quantitation of benzotriazole complexes to study their behavior in coordinating free copper, cobalt, and zinc ions and metal-containing enzymes, where their ions are integral to the active site specifically superoxide dismutase and cobalamin.

3. Material and Methods

3.1 Polarography

Polarographic and voltammetric measurements were performed with a CV-50W Voltametric analyzer fitted with a controlled growth mercury electrode. The multimode electrode was used in static mercury drop electrode (SMDE) mode. A three-electrode system was multiplexed with a platinum auxiliary electrode and an Ag/AgCl (3.0 M KCl) reference electrode. All potentials were relative to the Ag/AgCl electrode. A system scan rate of 5 mV s^{-1} was used with a drop time of 1.0 sec. For DPP, the pulse amplitude was 100 mV with a sample width of 17 msec.

3.2 Reagents

Britton-Robinson buffer was the supporting electrolyte to determine the stability of methylbenzotriazoles interactions with Cu, Co, and Zn. Phosphate buffered saline (pH 7) was used as supporting electrolyte to observe methylbenzotriazole complexation interactions with superoxide dismutase and vitamin B₁₂.

All chemicals used for polarographic analysis were of analytical grade and in high purity (>97%). Copper, and zinc sulfate, and hexamine cobalt chloride (for DPP analysis) were obtained from Fisher Scientific (Pittsburgh, PA, USA). When added to the solution used in this study, these salts liberated Cu (II), Zn (II), and Co (III) as the predominant forms of these metals. Superoxide dismutase and Vitamin B₁₂ were obtained from ICN Biomedicals, Inc. (Aurora, OH, USA). Britton-Robinson buffers were prepared in the polarographic vessel, by mixing 0.04 M phosphoric acid, 0.04 M acetic acid and 0.04 M Boric acid to give a pH 2.5. MeBT standards were obtained from PMC Specialty Group Inc. (Cincinnati, OH, USA) or bought from Sigma-Aldrich Corporation (St Louis, MO).

3.3 Procedures

3.3.1 Differential Pulse Polarography (DPP).

A 15 ml supporting electrolyte solution was pipetted into the voltammetric cell, and a metal ion (Co, Zn, or Cu) was added to give a concentration between 10^{-4} - 10^{-5} M. The ligand (methylbenzotriazole) was then added in increasing concentrations to a maximum of 3.0×10^{-3} M. The pH and the temperature were maintained at pH=2.5; room temperature respectively. The shift in the half wave potential was recorded after each addition and polarograms were compiled. All DPP measurements were made in triplicate, and each complexation experiment was independently replicated at least three times. Since commercially significant application of corrosion inhibitors often include a mix of 4- and 5- methylbenzotriazoles, a consecutive set of complexation experiments were extended with each isomers alone, as well as with the mix of isomers (ratio of 4-MeBT to 5-MeBT was 45:55) that are commercially available in the North American market.

4. Results and Discussion

4.1 Reversibility

DeFord-Hume analysis is based on an assumption that the complexation observed on the electrode surfaces is reversible, which is the case for many benzotriazole-metal interactions. According to a widely accepted metric developed by Parry and Osteryoung (1965), the reversibility of a polarographic wave is most accurately assessed by its half peak width [24]. These researchers have derived a general expression for the half peak width of DPP polarogram, which predicts a value of 62mV for a fully reversible two electron reduction at an applied potential pulse of 50 mV. In the present work, DPP polarograms in Britton-Robenson buffer had half peak widths of $62 \pm 2\text{ mV}$ for both free copper, cobalt, and complexed copper and cobalt, indicating a high degree of reversibility. To confirm the reversible reduction, reversibility was further tested using Tomes criterion [25]. According to this criterion, $E_{3/4}-E_{1/4}$ for a two electron reduction process resulted in a 29 mV response with applied pulse at 50 mV. $E_{3/4}-E_{1/4}$ represents the potential at $3/4$ and $1/4$ of the diffusion current respectively. In this present DPP work, the $E_{3/4}-E_{1/4}$ for Cu (II) and Co (II) was found to be 30 ± 1 and 29 ± 1 mV respectively when applied pulse at 50 mV.

4.2 Cu (II): 4, and 5-Methylbenzotriazole Systems

A relatively large potential shift was observed when 4-MeBT and 5-MeBT were added separately to solution containing Cu (II); this shift pattern validated the use of DPP for formation constant determination. Atypical DPP of those associated with a copper-4-Methylbenzotriazole and 5-Methylbenzotriazole systems is shown in Figure 1. The conditional stepwise stability constants (i.e. non-saturated copper complexes) as well as the overall (conditional) stability constant β_2 for Cu (II) were determined.

Polarographic results obtained for Cu (II): 4 and 5-methylbenzotriazole systems are presented in Table 1 and in Figure 1. Plotting $F_0(X)$ vs. [MeBT] using the data in Table 1 yielded a rising curves with an intercept of β_0 , which was equal to one for both systems. A first derivative of the previous plot ($F_1(X)$ vs. [MeBT]) yielded a straight line indicating that copper ions are coordinated with benzotriazole ligands at a maximum stoichiometric ratio of 2 for both systems as well. Regression analysis for Cu (II): 4-Methylbenzotriazole and 5-Methylbenzotriazole systems showed that the intercept of the first derivative plot, $F_1(X)$ corresponded to a value of β_1 (first complex stability) that approximately equal to 3.0×10^{10} and 6.0×10^{12} respectively. The slope corresponded to a value of β_2 (overall stability constants) approximately equal to 5.0×10^{13} and 7.0×10^{15} respectively. A confirmation for the value for β_2 was provided by the intercept of the second derivative plot, $F_2(X)$ vs. [MeBT], which yielded a straight line parallel to the [MeBT] axis, indicating that the highest ligand: metal ratio of Cu-4 or 5-MeBT interactions was 2. The intercept was found to equal 4.6×10^{13} and 7.0×10^{15} respectively.

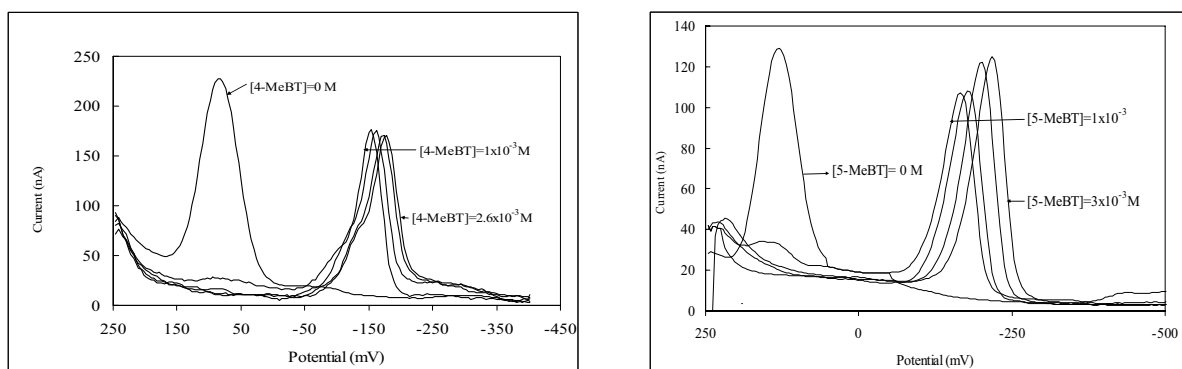


Figure 1: Differential pulse polarogram of Cu (II): methylbenzotriazole systems. A significant shift in the potential occurred in both systems (4-methylbenzotriazole (left), 5-methylbenzotriazole (Right)) systems as a result of an increase in the benzotriazole. $[Cu^{+2}]$ is equal to $5.3 \times 10^{-5} M$.

Table 1: Polarographic data for a Cu (II):4 and 5-methylbenzotriazole systems

Added Ligand Conc. (M)	ΔE_p (mV)	I_s/I_c	$F_0(CL)$	$F_1(CL)$	$F_2(CL)$
Cu(II):4-methylbenzotriazole system					
0.00E+00	0	-	1.00E+00	-	-
1.12E-03	235	1.03	8.97E+07	8.01E+10	4.47E+13
1.30E-03	240	1.02	1.24E+08	9.56E+10	5.06E+13
1.47E-03	241	1.03	1.42E+08	9.63E+10	4.51E+13
1.64E-03	244	1.05	1.73E+08	1.06E+11	4.6E+13
1.98E-03	248	1.03	2.30E+08	1.17E+11	4.39E+13
2.61E-03	255	1.08	3.94E+08	1.51E+11	4.62E+13
2.77E-03	257	1.02	4.30E+08	1.55E+11	4.53E+13
Cu (II):5-methylbenzotriazole system					
0.00E+00	0	-	1.00E+00	-	-
1.09E-03	292	2.05	1.47E+10	1.34E+13	6.77E+15

1.44E-03	298	1.78	2.30E+10	1.60E+13	6.99E+15
1.77E-03	304	1.69	3.25E+10	1.84E+13	7.02E+15
1.93E-03	306	1.63	3.69E+10	1.91E+13	6.80E+15
2.09E-03	308	1.60	4.21E+10	2.01E+13	6.76E+15
2.40E-03	312	1.54	5.47E+10	2.28E+13	6.98E+15
2.55E-03	314	1.50	6.22E+10	2.43E+13	7.18E+15

The maximum coordination number was confirmed for both systems using Lingane method [23]. Half wave potential shifts in response to Log [4 and 5-MeBT] shown in Figure 2 confirmed the previous

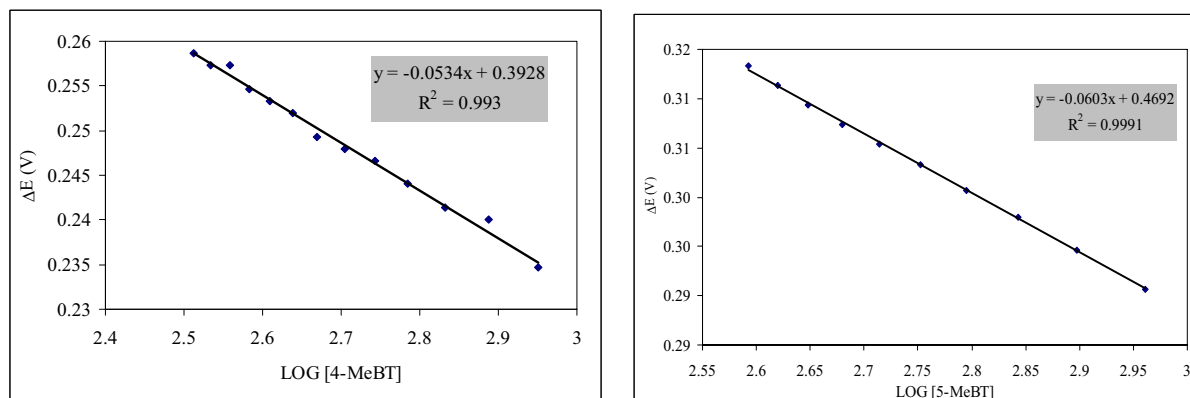


Figure 2: Half wave potential shift for the reduction of Cu (II) in response to increasing concentrations of 4-methylbenzotriazole (left) and 5-methylbenzotriazole (right) systems. pH=2.5; T=25°C. Symbols represent the averages of triplicate observations; error bars represent standard deviation. results with the slope approaching the maximum ligand number of 2.

4.3 Zinc (II)-Methylbenzotriazole System

No significant potential shifts were observed when increasing 4-or 5-methylbenzotriazole masses were added to zinc-containing solutions. Those results indicating that either weak, no complexes formed, or DPP could not be used in this application. Since DPP was unsuitable for formation constant determination of Zn(II): MeBT complexes, further electrochemical characterization was not required for zinc-MeBT interactions.

4.4 Co (III): 4 and 5-Methylbenzotriazole Systems

A potential shift was observed when 4 and 5-MeBT were added to solution containing Co (III); this shift again validated the use of DPP for formation constant determination. Figure 3 shows the typical polarograms associated with cobalt: 4 and 5-methylbenzotriazole systems respectively after the incremental additions of MeBT. As before, conditional stepwise stability constants as well as the overall stability constant β_2 for Co (III) were determined. Polarographic results obtained for Co (III): 4 and 5-methylbenzotriazole systems are presented in Table 2 and Figure 3. Plotting $F_0(X)$ vs. [MeBT] yielded a rising curve with an intercept of β_0 , which was equal to one. Also, first derivative of the previous plot, $F_1(X)$ vs. [MeBT], yielded a straight line indicating that copper ions are coordinated with benzotriazole ligands at a maximum stoichiometric ratio of 2.

Regression analysis for both 4 and 5-methylbenzotriazole systems indicated that the intercept of the first derivative plot, $F_1(X)$ corresponded to a value of β_1 approximately equal to 1.0×10^6 and 9.0×10^5 respectively while the slope of this line corresponded to a value of β_2 approximately equal to 2.0×10^8 and 5.0×10^7 respectively as well. A confirmation for the value for β_2 was provided by the intercept of the second derivative plot, $F_2(X)$ vs. [MeBT], which yielded a straight line parallel to the [MeBT] axis,

indicating that the highest ligand: metal ratio of Co-MeBT interactions was 2 for both systems. The intercept was found to equal 2.4×10^8 and 5.0×10^7 respectively.

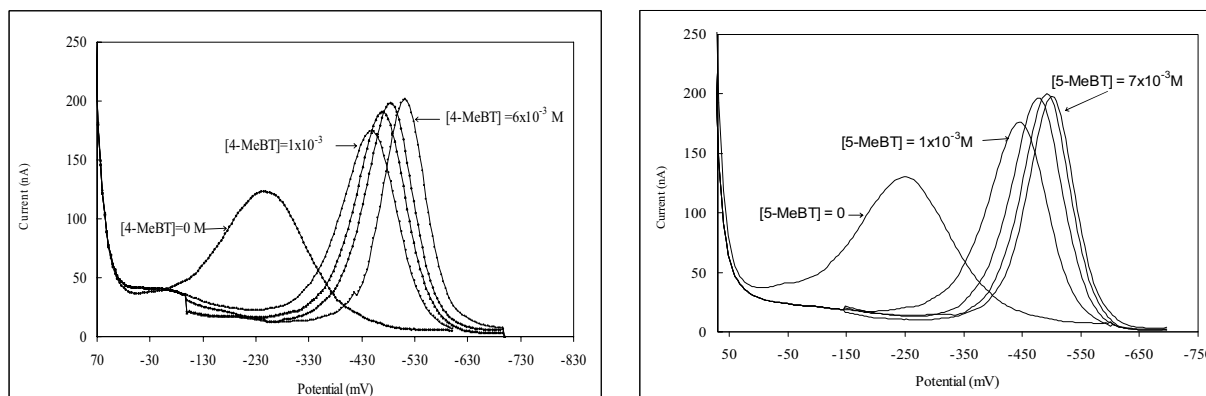


Figure 3: Differential pulse polarogram of Co(III): methylbenzotriazole systems. A significant shift in the potential occurred in both systems (4-methylbenzotriazole (left), 5- methylbenzotriazole (Right)) systems as a result of an increase in the benzotriazole. [Co III] is equal to 1.5×10^{-5} M.

Table 2: Polarographic data for a Co(III): 4 and 5-methylbenzotriazole systems

Added Ligand Conc. (M)	Ligand	ΔE_p (mV)	I_s/I_c	$F_0(\text{CL})$	$F_1(\text{CL})$	$F_2(\text{CL})$
Co(III):4-methylbenzotriazole system						
0	0	-	-	1.00E+00		
1.095E-03	200	0.59		1.42E+03	1.30E+06	2.71E+08
2.094E-03	224	0.51		3.16E+03	1.51E+06	2.42E+08
3.008E-03	240	0.47		5.44E+03	1.81E+06	2.69E+08
4.621E-03	256	0.42		9.12E+03	1.97E+06	2.11E+08
5.337E-03	264	0.41		1.21E+04	2.26E+06	2.36E+08
6.618E-03	272	0.40		1.59E+04	2.39E+06	2.11E+08
Co(III):5-methylbenzotriazole system						
0.00E+00	0			1.00E+00		
1.07E-03	188	0.63		9.61E+02	8.97E+05	-5.57E+06
2.94E-03	228	0.51		3.17E+03	1.08E+06	5.96E+07
3.76E-03	236	0.49		4.12E+03	1.10E+06	5.13E+07
5.22E-03	248	0.45		6.09E+03	1.17E+06	5.08E+07
5.87E-03	252	0.44		6.92E+03	1.18E+06	4.72E+07
7.03E-03	260	0.42		9.14E+03	1.30E+06	5.64E+07

The maximum coordination number was confirmed for both systems using Lingane method [23]. Half wave potential shifts in response to Log [4 and 5-MeBT] shown in Figure 4 confirmed the previous results with the slope approaching the maximum ligand number of 2 for both systems.

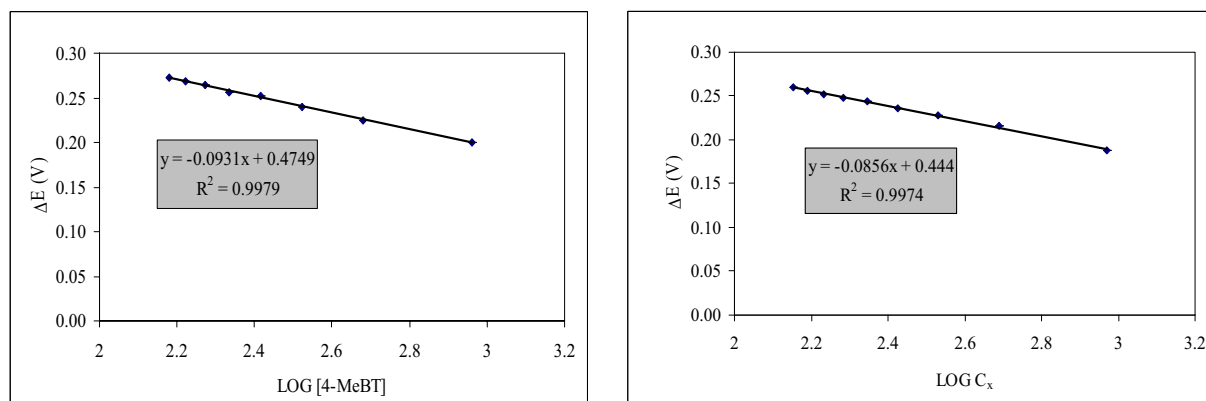


Figure 4: Half wave potential shift for the reduction of Co (III) in response to increasing concentrations of 4-methylbenzotriazole (left) and 5-methylbenzotriazole (right) systems. pH=2.5; T=25°C. Symbols represent the averages of triplicate observations; error bars represent standard deviation.

Summary of stability constants for methyl benzotriazole isomers complexes with Cu(II), Co(III) and Zn(II) derived from DeFord-Hume and Lingane polarographic analysis is shown in Table 3.

Table 3: Stability constants for 4-methylbenzotriazole and 5-methylbenzotriazole complexes with Cu(II) and Co(III) derived from DeFord-Hume and Lingane polarographic analysis.

Concentration (M)			Method			
System	Metal	MeBT	Lingane	DeFord-Hume		Max. coordination number
			Log β_n	Log β_1	Log β_2	
Cu(II):5-MeBT	5.2×10^{-5}	$0 - 3.0 \times 10^{-3}$	15.9	12.8	15.8	2
Cu(II):4-MeBT	5.3×10^{-5}	$0 - 3.0 \times 10^{-3}$	13.7	10.5	13.7	2
Co(III):5-MeBT	1.5×10^{-5}	$0 - 7.0 \times 10^{-3}$	7.8	6.0	7.7	2
Co(III):4-MeBT	1.5×10^{-5}	$0 - 7.0 \times 10^{-3}$	8.0	6.0	8.3	2
Zn(II):5-MeBT	5.5×10^{-5}	$0 - 2.0 \times 10^{-3}$	-	-	-	-
Zn(II):4-MeBT	5.5×10^{-5}	$0 - 2.0 \times 10^{-3}$	-	-	-	-

4.5 Interactions of Superoxide Dismutase with MeBT

An Addition of benzotriazole isomers to solutions containing active superoxide dismutase (SOD) causes a potential shift (Figure 5). This shift is related to the formation of a complex between the copper metal in the SOD enzyme, and methylbenzotriazole isomers. The behavior was the same as that between free copper metal and MeBT, meaning that the complex formed between SOD and MeBT is strong nearly as strong as that between MeBT and free Cu and as judged by -200 mV potential shifts observed upon MeBT addition to solution containing active SOD. However, unlike MeBT interactions with free copper, the reduction was found to be irreversible once the MeBT was bound to SOD. Such that DPP could not be used to calculate the conditional stability constant.

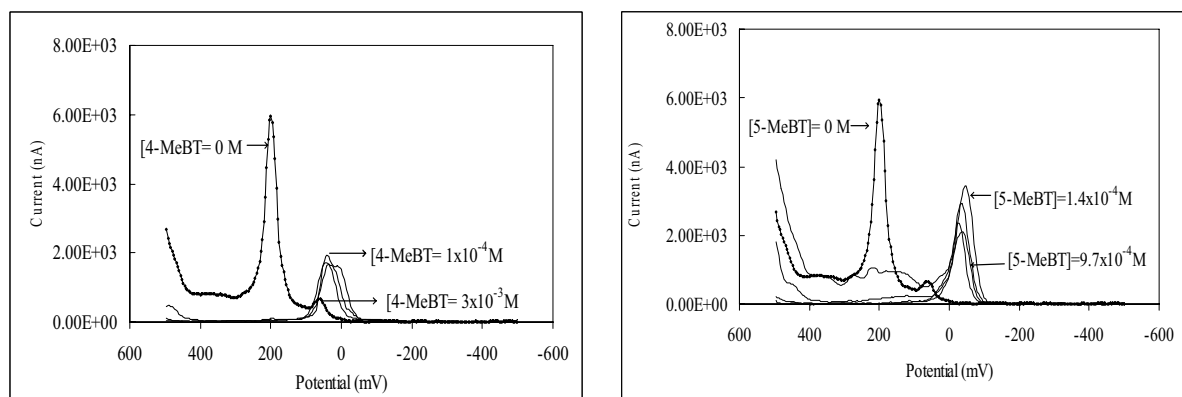


Figure 5: Differential pulse polarogram of SOD: 4-methylbenzotriazole (Left), 5-methylbenzotriazole (Right) systems, showing the potential shift and streaming current reduction resulting from the addition of increasing amount of benzotriazole. $[\text{Cu(II)}]$ in SOD = $6.0 \times 10^{-7} \text{ M}$.

4.6 Interaction of Vitamin B₁₂ with MeBT

The interaction between methylbenzotriazole and free cobalt ions was observed, however, no significant shift in potential occurred with the increase of the MeBT concentration in a solution containing cobalamin (Figure 6). Decreasing in the current were observed, and the decrease were proportional to incremental masses of MeBT added (Figures 7). The results suggest that a MeBT-cobalamin complex indeed formed, and the amount formed was proportional to the amount of ligand added; however, the inability to measure a potential shift indicated that DPP techniques are unsuitable for formation (stability) constant determination between MeBT and cobalamin.

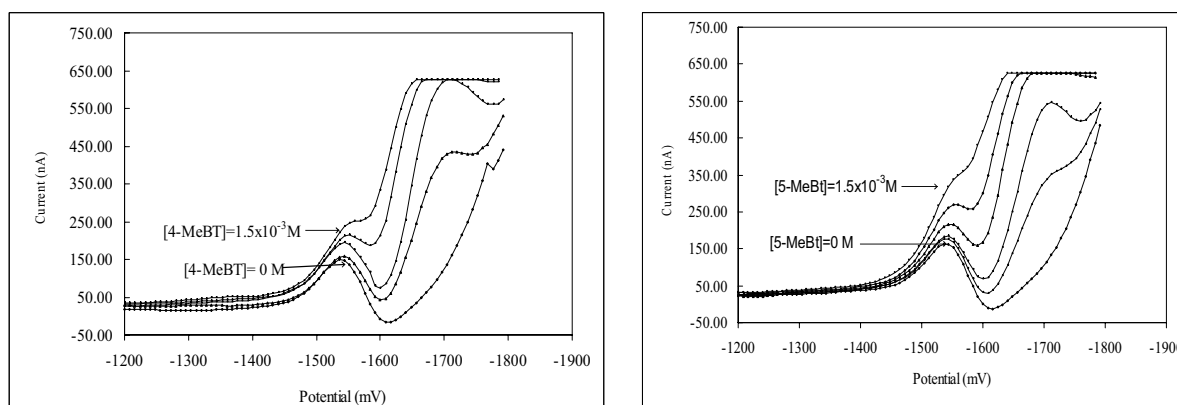


Figure 6: Differential pulse polarography of Vitamin B₁₂: 4-methylbenzotriazole (left) and 5-methylbenzotriazole (right) systems showing streaming current reduction resulting from the addition of increasing amounts of benzotriazole. $[\text{Co(III)}]$ in Vitamin B₁₂ = $5.7 \times 10^{-7} \text{ M}$.

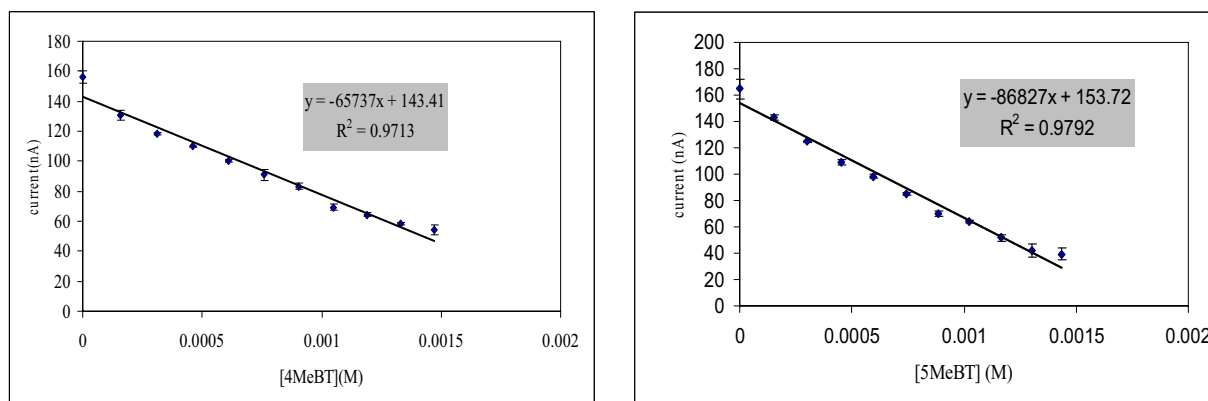


Figure 7: Streaming reduction current response of vitamin B₁₂ (Co(III)) in response to the addition 4-MeBT (left) and 5-MeBT (right) at pH 7 (phosphate buffer). Results are averages of 3 independent observations. Error bars represent 1 standard deviation.

5. Summary and Conclusion

Results from this study suggested that commercially significant methylbenzotriazole isomers are able to form a very strong complexes with free copper and cobalt for both benzotriazole isomers tested. The conditional stability constants were approximately 10^{15} and 10^8 respectively for copper and cobalt. Complexation of MeBT with Zn (II) could not be detected by differential pulse polarographic techniques (DPP). It was found that Lingane and DeFord-Hume analysis were in a very good agreement under all conditions tested. The differences did not exceed $10^{0.3}$ difference.

Methylated benzotriazoles coordinated and bound copper in the active site of a superoxide dismutase, as well as with the redox-active cobalt conjugated in a corrin group in the ubiquitous co-enzyme cobalamin (vitamin B₁₂). The strength of binding (i.e. stability constant) could not be directly determined by polarography; however, the reduction spectra suggest that like free copper and cobalt, the stability constants and associated binding energy is quite large. This type of strong selected coordination may represent a novel mode of toxicity associated with corrosion inhibitors: changing the redox potential of a transition metal responsible for electron transfer in the active site of essential oxido-reductase enzymes.

While concurrent assays of benzotriazole-enzyme coordination and enzyme activity were not experimentally feasible during these investigations, the large negative potential shift observed when benzotriazoles were added to active enzyme-containing solutions suggested that once it bound, some catalytic inhibition would result.

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