



INTERACTION OF THE FUNGICIDE TEBUCONAZOLE WITH HUMAN SERUM ALBUMIN: A PRELIMINARY STUDY

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ABSTRACT

The interactions between the fungicide tebuconazole and human serum albumin were investigated using fluorescence and circular dichroism spectroscopies. The experimental results showed that the fluorescence quenching of the protein by the tebuconazole molecule was a result of the formation of a ligand-protein complex with a binding constant of $8.51 \times 10^3 \text{ L.mol}^{-1}$ and the number of binding sites in the macromolecule was close to 1. These findings demonstrated the fact that although the binding affinity of tebuconazole to the protein may be slight, it was very similar to other triazole fungicides. In addition, tebuconazole stabilized the α -helical secondary structure of the human serum albumin due to the increase of the α -content in the protein macromolecule.

Key words: association constant; fluorescence spectroscopy; human serum albumin; interaction; tebuconazole

INTRODUCTION

Tebuconazole [1-(4-chlorophenyl)-4,4-dimethyl 3-(1,2,4-triazol-1-ylmethyl) pentan-3-ol] (Fig. 1) is a representative of triazoles that are a class of fungicides largely used in agriculture as crop protection products [3]. Their antifungal activity is due to their ability to inhibit the P450 enzyme, which blocks the conversion of lanosterol to ergosterol causing disruption of the fungal cellular wall [5]. But the inhibition potency of these triazole fungicides including tebuconazole (TB) is not limited to fungi; they may also inhibit other P450-mediated activities resulting in various adverse effects [21].

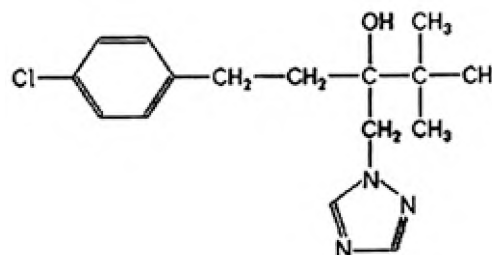


Fig. 1. The chemical structure of tebuconazole

TB is the triazole fungicide, which is applied on a number of crops such as grapes, rice, fruits, and vegetables because of its broad-spectrum antifungal activity. This fungicide had been classified by the US EPA as Group C-Possible Human Carcinogen [27]. TB is persistent in soils and presents moderate mobility [6]. It is classified as toxic to aquatic organisms and may cause long-term adverse effects in the aquatic environment [2]. The presence of TB in stream water has increased in recent years [18] and its concentrations detected in surface waters were up to 175–200 pg.l^{-1} [7]. The results obtained by Liang et al. [17] indicated that exposure to TB could alter thyroid hormone levels as well as gene transcription in zebrafish larvae. Commercial formula Orius 25EW containing 25 % of tebuconazole applied in agriculture as a leaf and ear spray pesticide has been studied by Holeckova et al. [12]. In this study, significant chromosomal aberrations in lymphocytes induced by the fungicide were discovered. Furthermore, TB-based fungicide induces sister chromatid exchange in bovine peripheral lymphocytes [26]. What's more, TB was found in human beings with the maximal concentrations of 19.2 pg.l^{-1} and 2.22 ng.kg^{-1} in urine and hair samples, respectively, from farm workers [9, 22].

In fact, because so little is known about the long-term toxicity of TB and other triazoles to vertebrates at this time, it is not possible to ascertain whether these molecules present a risk for mammals chronically exposed to low doses. As a consequence, preliminary studies of interactions, in a broad sense, between TB and several proteins of interest, are one of the necessary steps toward an increased understanding of TB's toxicity on mammals.

Human serum albumin (HSA) is the most abundant plasma protein and contributes significantly to many transport and regulatory processes. It forms about 60 % of the mass of human plasma proteins with a typical concentration of 40 mg.ml^{-1} in the bloodstream [24, 10]. The protein binds to a wide variety of substrates such as metals, fatty acids, amino acids, hormones and an impressive spectrum of drugs [19].

Recently, several studies have been carried out to examine the toxic effects of pesticides at the protein level [23, 4, 32, 30] but information about the possible impact of triazoles on plasma proteins is still limited [29, 34]. At the same time, a binding of pesticides including triazoles to plasma proteins has toxicological importance as it can significantly affect their distribution and excretion in the

organism [25]. When these fungicides penetrate into the bloodstream, they may bind to plasma proteins and subsequently induce some alternations of the protein structure and function.

The aim of this study was to obtain additional information about the interactive properties of TB in relation to plasma proteins with regard to the above mentioned frequent application of TB as a pesticide. These results may provide basic data for clarifying the binding mechanisms of TB with HSA and be helpful for food security and human health when TB is applied as an antifungal agent. Fluorescence and far UV circular dichroism spectroscopy (far UV CD) were used for a determination of the binding constant, mode of interaction between TB and human serum albumin molecules, and the effect of fungicide on the secondary structure of the HSA molecule.

MATERIALS AND METHODS

Tebuconazole (CAS Number 107534-96-3) was obtained from Sigma, and its grade was analytical standard. HSA (fatty acids free, globulin free, purity no less than 99 %) was purchased from Sigma and was used without further purification. To prepare stock solutions, TB was dissolved in 100 % ethanol in a concentration of $10^{-3} \text{ mol.l}^{-1}$ and a stock solution of HSA (concentration $5 \times 10^{-4} \text{ mol.l}^{-1}$) was prepared in Tris-HCl (0.05 mol.l^{-1} Tris + 0.1 mol.l^{-1} NaCl) buffer, pH 7.4. Phosphate buffer (0.02 mol.l^{-1} , pH 7.4) was used for the CD spectroscopic measurements. Tris(hydroxymethyl)aminomethane, NaCl, HCl, and other reagents were all of analytical purity.

Fluorescence measurements were performed using a spectrofluorometer SHIMADZU RF 5301 PC in 1 cm quartz cuvette. The excitation wavelength was 295 nm and fluorescence was collected at 300–500 nm emission wavelengths using 5 nm/5 nm slits.

Complexes of TB/HSA for fluorescence spectroscopy were prepared by titration of TB into $2 \times 10^{-6} \text{ mol.l}^{-1}$ HSA to have a final concentration of TB from $2 \times 10^{-6} \text{ mol.l}^{-1}$ to $32 \times 10^{-6} \text{ mol.l}^{-1}$.

Far UV CD spectroscopy experiments were carried out by means of a CD spectrometer Jasco J-815 in 0.1 cm quartz cuvette at room temperature and constant nitrogen flush. The spectra of HSA in the presence of TB were recorded in the range 200–270 nm with a scan rate of 50 nm.min^{-1} .

Three scans were accumulated for each spectrum, taking the average as the final data.

Complexes of TB/HSA for CD spectroscopy were prepared by the titration of TB into 3.75×10^{-6} mol.l⁻¹ HSA to obtain a final concentration of TB from 3.75×10^{-6} mol.l⁻¹– 1.875×10^{-5} mol.l⁻¹. The Tris-HCl buffer was replaced by phosphate buffer (0.02 mol.l⁻¹, pH 7.4) to eliminate the influence of Cl⁻ ions. The helical content of free and bound HSA was calculated from the mean residue ellipticity (MRE) values at 209 nm using the following equation

$$\% \alpha\text{-helix} = \frac{-\text{MRE}_{209\text{nm}} \times 4000}{33000 - 4000} \times 100$$

Graphical presentations were evaluated by software Origin, versions 6.0 and 8.0.

RESULTS

Fluorescence spectroscopy is an effective method to study the interactions between small molecules and bio macromolecules. Fluorescence quenching refers to any pro-

cess, which decreases the fluorescence intensity of a sample. The fluorescence of fluorophores within the HSA macromolecule may change when HSA interacts with other molecules, which could be reflected in the fluorescence spectra of HSA in the UV region. The changes in emission intensity value, and wavelength of emission maximum, respectively, are evaluated at experimental processing. Whereas the fluorescence intensity is influenced by a change in the polarity of the microenvironment of bio macromolecule as well as movement of charged groups in the vicinity of the fluorophores inside, hydrophobic changes in the microenvironment primarily dictate the shift in the emission maximum [14]. The shift in the emission maximum is a good index to evaluate any alternation in the hydrophobicity of the binding region [8]. Our emission fluorescence spectra of HSA in the absence and presence of TB are shown in Figure 2. It can be seen that the fluorescence intensity of the HSA decreases as a result of the increasing concentration of TB. This result points to moderate fluorescence quenching effect (Fig. 2 inset) but at the same time a remarkable deformation of emission spectra can be seen (Fig. 2). It looks like there was a blue shift observed at the highest TB concentration used in this study, which should point to any

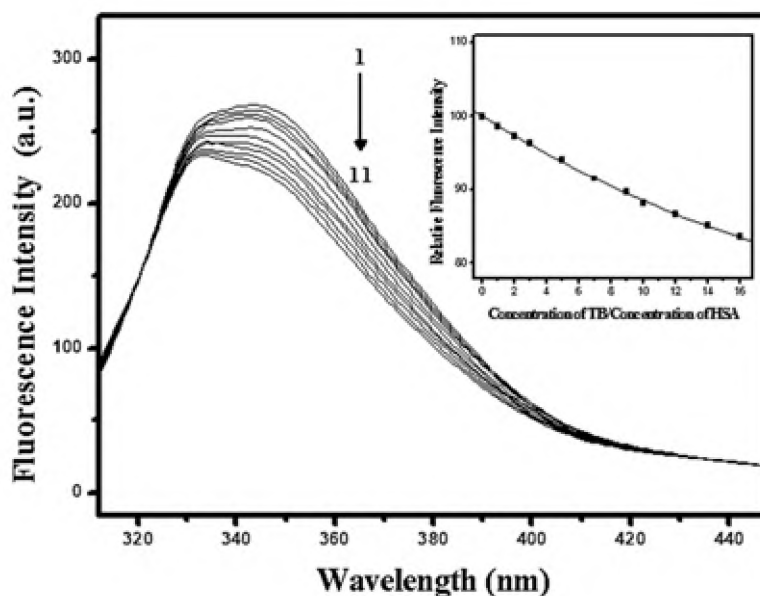


Fig. 2. Effect of tebuconazole on the fluorescence spectra of HSA

c(HSA) = 2 pmol.l⁻¹. Lines 1-11: c(TB) = 0; 2.0; 4.0; 6.0; 10.0; 14.0; 18.0; 20.0; 24.0; 28.0; 32.0 pmol.l⁻¹, respectively.

λ_{exc} = 295 nm; λ_{em} = 300–500 nm; pH = 7.4; T = 298 K

Inset: Fluorescence decrease of HSA upon TB binding at emission wavelength 344 nm

alternation in the hydrophobicity of the binding region of the HSA. However, the deformation of the emission spectrum in Fig. 2 cannot be regarded as the blue shift in the emission maximum but it probably presents a sum of two individual fluorescence spectra with maximum at 332 nm, and 351 nm respectively. Subsequently completed spectral deconvolution (not shown) has proven our above mentioned assumption. Considering a chemical structure of TB and hydrophobic cavity within the HSA macromolecule, two different modes of interaction between TB and the HSA can be assumed. At low concentration of TB, the TB molecules quench intrinsic fluorescence of HSA due to the interaction with the hydrophobic part of the protein showing a decrease in fluorescence intensity at 351 nm. On the other hand, at higher concentrations of the TB in complex, some TB molecules are inert and they enable the HSA macromolecule to have unchanged emission spectrum at 332 nm. Finally, we can assume there are two populations of the HSA macromolecules as a consequence of the interaction with TB at higher ratios.

A variety of molecular interactions can result in quenching, including excited-state reactions, molecular rearrangements, energy transfer, ground-state complex formation and collisional quenching [15]. The quenching mechanism can be described by the Stern-Volmer equation:

$$F_0/F = 1 + k_q \tau_0 [Q] = 1 + K_{SV} [Q] \quad (1)$$

Where F_0 and F are the fluorescence intensities of the

protein in the absence and presence of the quencher (TB), respectively. $[Q]$ is the concentration of the quencher, k_q is the bimolecular rate quenching constant, τ_0 is the average lifetime of the biomolecule without quencher about 10 ns for most biomolecules [16] and K_{SV} is the Stern-Volmer constant. The inverse value of K_{SV} represents the quencher (TB) concentration at which 50 % of the fluorophore intensity (HSA) is quenched.

The curve of $(F_0/F)-1$ versus $[Q]$ is shown in Fig. 3a. The corresponding k_q and K_{SV} constants for the TB/HSA complex were obtained by the fitting of the graphical representation of the Stern-Volmer equation. The quenching constants are summarized in Table 1.

Identification of binding parameters

If we assume static quenching then the association constant (K_A) and the number of binding sites (n) in the bio macromolecule can be calculated by the following equation [31].

$$\log(F_0/F-1) = \log K_A + n \log [Q] \quad (2)$$

Fitting the plot of $\log(F_0/F-1)$ versus $\log [Q]$ (so called Hill plot) for the interaction between the HSA and tebuconazole (Fig. 3b), the association constant and number of binding sites were obtained (Table 1).

To obtain more information on the binding of tebuconazole to HSA, circular dichroism spectroscopy was used to study the secondary structure of HSA and TB/HSA complex. CD is a sensitive technique to monitor the conforma-

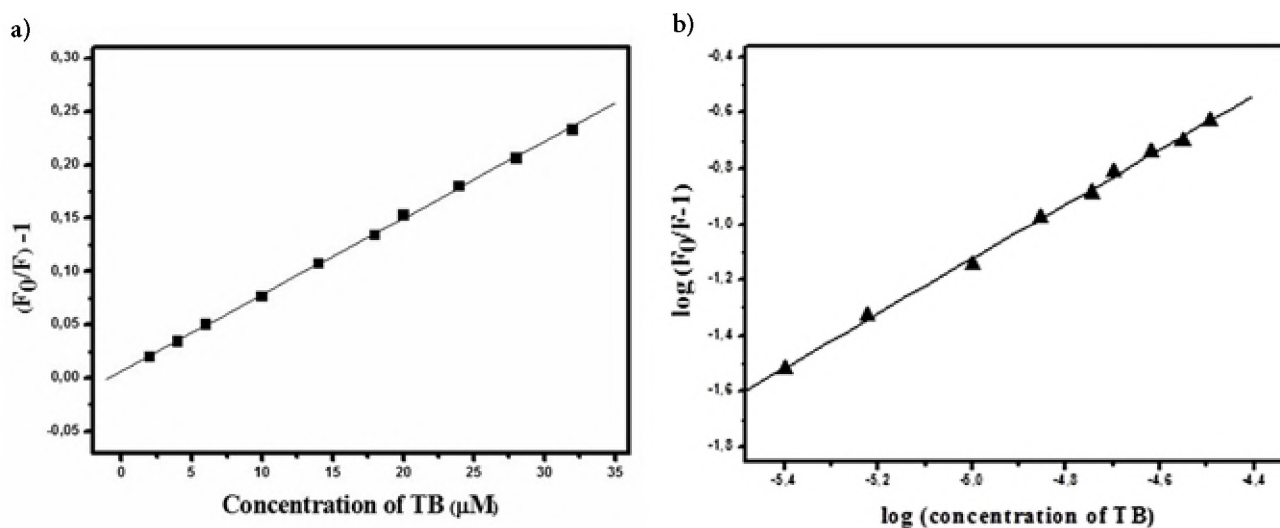


Fig. 3. a) Stern-Volmer representation of the fluorescence quenching of the HSA by tebuconazole;
b) Hill plot for the determination of the binding parameters of the HSA interaction with TB
 $c(\text{HSA}) = 2 \times 10^{-5} \text{ mol.L}^{-1}$; $\lambda_{\text{ex}} = 295 \text{ nm}$; $\lambda_{\text{em}} = 344 \text{ nm}$, pH = 7.4; T = 298 K

Table 1. Bimolecular rate quenching constant (kq), Stern-Volmer quenching constant (KSV), association constant (KA) and number of binding sites (n) at the physiological conditions

T (K)	kq [l.mol ⁻¹ .s ⁻¹]	KSV [l.mol ⁻¹]	KA [l.mol ⁻¹]	n
298	7.26 X 10 ¹¹	7.26 X 10 ³	8.51 X 10 ³	1.01

tional changes in proteins. Far UV CD measurements were performed in the presence of TB at different concentrations but the CD spectra stayed identical for the final concentration ratio 5/1 (Fig. 4).

As can be seen from Fig. 4, the CD spectra of HSA and the complex, respectively, display two negative bands in the ultraviolet region at 209 and 223 nm, characteristic of the α -helical structure of protein. The reasonable explanation is that the negative peak at 209 nm is contributed to $n \rightarrow n^*$ transition and 223 nm peak is contributed to $n \rightarrow n^*$ transfer for the peptide bond of α -helix [33].

As shown in Figure 4, TB alone (dotted line) does not show any optical activity in this region. The addition of TB to HSA leads to an increase in the CD signal without significant shift of the peaks indicating that the binding of TB to HSA induces an increase in the α -helical content of HSA. The α -helix content of HSA increased from 55 % to 60 % upon TB binding at a molar ratio of TB/HSA of 1/1 and

5/1, implying that the TB binding results in a conformation change that stabilizes the HSA structure by increasing its α -helical content. We assume that the increase of the α -helical content of HSA is realized at the expense of the random coil content when TB binds to the protein.

DISCUSSION

A better understanding of the interaction of the TB and other fungicides with various possible cellular targets is essential for the determination of their function in biological systems. The present contribution was focused on the results obtained in the study of TB associations with serum albumins represented by HSA.

The value of the association constant (Table 1) of the TB/HSA complex induced a slight binding affinity between the TB and the HSA. The interaction of four triazole fungicides (triadimefon, imazalil, myclobutanil, penconazole) with HSA has been reported in a comprehensive study by Zhang et al. [34]. The association constants determined by fluorescence quenching in this study range from 3.96×10^3 l.mol⁻¹ (triadimefon) to 8.47×10^3 l.mol⁻¹ (penconazole) [34]. Our association constant for the TB/HSA complex (8.51×10^3 l.mol⁻¹) falls into this interval very well. The number of binding sites for TB, which is close to 1 (Table 1) corresponded with those obtained by Zhang

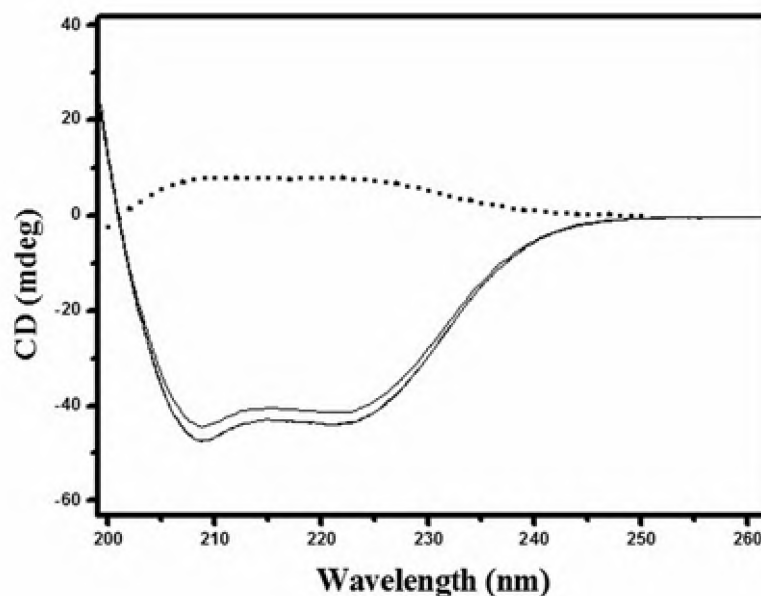


Fig. 4. Far UV CD spectra of HSA in absence and presence of TB
c(HSA) = 3.75 pmol.l⁻¹, c(TB) = 0; 3.75; 18.75 pmol.l⁻¹, phosphate buffer pH = 7.4

et al. [34]. Considering a chemical structure of all of the estimated triazole fungicides we may assume that both tebuconazole and penconazole molecules look similar. This fact can be one of many other reasons explaining their very similar binding affinity expressed by the association constants.

Comparing the binding affinities of other pesticides to HSA, we can consider the studies by Wang et al. [29] and Wang et al. [30]. They used the spectroscopic approach to find association constants for imidacloprid/HSA and thiacloprid/HSA. Both molecules represented the neonicotinoid insecticides currently being used in agriculture. Comparing the association constants of triazole and neonicotinoid pesticides we can declare that the binding affinity of triazole fungicides, including tebuconazole to HSA, is lower than those of neonicotinoides. But the differences are not very expressive.

It has previously been reported that the binding of certain ligands such as ciproflaxin [1], virstatin [13], phycocyanobilin [20] may cause a conformational change in HSA with an accompanying increase in its α -helical content and protein structural stabilization. This phenomenon is quite striking for protein-ligand systems with high affinities, e.g., binding of biotin to streptavidin causes the disappearance of the band arising from unordered structure in FT-IR spectra [11]. Surprisingly, an increase in the TB concentration causes the CD signal of the HSA to be increased, which indicates an increase of the helical secondary structure content in the HSA macromolecule. On the contrary, with the above mentioned knowledge about the tendency to increase the helical content in high binding affinity ligands, the binding affinity of TB to HSA is only slight. But Wang et al. [29] have obtained the same result for a slightly bound imidacloprid to HSA. The increase in negative ellipticity as shown in Fig. 4 might be due to the shielding of the peptide strand in the HSA macromolecule due to the increase in hydrophobicity on binding with TB [28]. This conclusion agrees with the result of the fluorescence quenching experiment.

The present study investigated the interaction of the triazole fungicide tebuconazole with HSA by spectroscopic methods. TB can interact with HSA *in vitro* under simulated physiological conditions. Considering a static mechanism of fluorescence quenching of HSA by the TB, we have determined the association constant which expresses the binding affinity between both molecules. The value of this constant indicates a slight binding affinity in comparison to

other ligands, but it corresponds with the binding parameters of other triazole fungicides very well. Furthermore, the interaction of TB led to slight conformational changes of the HSA macromolecule.

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