Chapter IV
Mechanism of MT-Human Serum Albumin Interaction and MT-Induced Protein Damage
1. Introduction

Albumin represents 52-60% of the total plasma protein content and plays an important role in transport of endogenous ligands and environmental chemicals and/or xenobiotics mostly through the formation of non-covalent complexes at specific binding sites (Bertucci & Domenici, 2002; Sugio et al., 1999). Covalent modification of proteins can have profound effects on their biological activities. Well-known physiological examples include phosphorylation (Krebs & Beabo, 1979), carboxylation (Vermeer, 1990), methylation (Kim, 1996), glycosylation (Clarke, 1985) and fatty acylation (Towler et al., 1988). Most drugs bind reversibly to a number of binding sites on albumin (Peters, 1996), and there is evidence of conformational changes in protein induced by its interaction with low molecular weight drugs. These changes appear to affect the secondary and tertiary structure of albumin. (Hushcha et al., 2000). The development of computational models for prediction of drug pharmacokinetics is an area of current intense research in the pharmaceutical industry (Butina et al., 2002) and human serum albumin (HSA) has a central role in drug pharmacokinetics (Herve et al., 1994; Carter & Ho, 1994).

Apart from the benefits driven from the albumin-drug binding, interactions of xenobiotics with ‘critical’ cellular macromolecules is thought to be an important step in the events that lead to cellular injury (Boelsterli, 1993). Toxicological processes can also lead to covalent changes affecting protein structure and biological activity. Among them there are modifications of protein amino acids by xenobiotic chemicals or their metabolites (Boelsterli, 1993). Modifications in the structural and functional integrity of proteins in plasma membranes and membranes of intracellular organelles may trigger alterations in energy metabolism, affecting biological cell activities such as synthesis of ATP, signaling, regulation of biosynthetic and catabolic reactions, transport of metabolites and ions (Palmeira, 1997). Competition between xenobiotic and endogenous substances for binding to plasma proteins can strongly affect the disposition of both substances, with possible serious physiological consequences (Bertucci & Domenici, 2002). In some cases, binding of small molecules is responsible for the protective role of albumin, such as the binding of bilirubin from the spleen to the liver, or the binding of
exogenous toxins, reducing consequences from their toxicity (Bertucci & Domenici, 2002). The molecular interactions between albumin (HSA) and many drugs have been investigated successfully (Tang et al., 2006; Li et al., 2005). However, the binding of the pesticides to proteins has seldom been investigated. Owing to the wide application of herbicide, 2,4-D has been given special attention for its interaction with serum albumin (Purcell et al., 2001). However, it is rare to find any report on fungicide-albumin interaction and fungicide-induced protein damage.

This has prompted to investigate the MT-albumin interaction and MT-induced protein degradation, and to test the hypothesis that cellular protein degradation may be due to increased ROS generation during MT photoexcitation. No systematic study has been made till date to assess the quantitative binding of MT with serum albumin. In this study, human serum albumin (HSA) has been chosen as a model protein to investigate the (i) extent and nature of interactions of MT with HSA (ii) binding constant \( K_a \) and capacity \( n \) of HSA for MT, (iii) MT-induced conformational changes in protein, using the sensitive techniques such as fluorescence spectroscopy, circular dichroism and cyclic voltammetry.
2. Materials and Methods

2.1. MT-HSA fluorescence measurements
Fluorescence measurements were carried out on a Shimadzu spectrofluorophotometer, model RF5301PC equipped with RF 530XPC instrument control software, at 25 ± 0.1 °C, using a quartz cell of 1 cm path length. The fluorescence spectra were measured at a protein concentration of 3.0 μM with a 1 cm path length cell. Excitation and emission slits were set at 3 nm each. The emission spectra were recorded in 290-380 nm range and the excitation wavelength was set at 280 nm, while MT was almost non-fluorescent in this range. All the stock solutions were filtered through 0.45 μm Millipore filters prior to mixing, to minimize the inner filter effect (Chignell, 1972).

2.2. Quantitative analysis of MT-HSA interactions
Binding affinity of HSA with fungicide MT was determined according to the method of Levine (1977). In brief, to a fixed concentration (3.0 μM) of protein solution, varying amount of MT was added to obtain the desired molar ratios. The final volume in each set of (MT/HSA) experiment was adjusted to 3 ml with Tris-HCl buffer, pH 7.4. The fluorescence intensity values at the emission maxima (340 nm) were used to determine the relative fluorescence, considering the fluorescence intensity of control untreated protein as 100. The least square analysis of the initial linear points of relative fluorescence vs. MT-HSA molar ratio was used to determine the maximal quench, (m) (slope of the plot) following the equation 
\[ F = F_0 - mA \]; were \( F \) is the fluorescence intensity at MT to HSA molar ratio (\( R \)) and \( F_0 \) is the fluorescence intensity of protein at zero concentration of pesticide. Selecting the data points deviating from straight-line behavior, the fractional quench (\( Q \)) was determined, at each MT-HSA molar ratio (\( R \)). For an observed fluorescence intensity \( F \), the fractional quench (\( Q \)) was determined from the equation 
\[ Q = F_0 - F/maximal quench (m) \]. Fractional quenching (\( Q \)) was linearly related to MT binding: 
\[ [MT-HSA] / [HSA]_T = Qm \] where the [HSA]_T represents the total HSA concentration. Binding affinity (\( K_a \)) and binding capacity (\( n \)) were determined from the slope and X- axis intercept of the straight line on the Scatchard plot \( Q \) vs. \( Q/(R-Q) \) [HSA]_T (Levine, 1977).
2.3. Cyclic voltammetric analysis of MT-HSA binding

The redox potentials of the MT and MT-HSA were determined by cyclic voltammetry in aqueous medium containing 0.4 M KNO$_3$, as a supporting electrolyte, at room temperature. Cyclic voltammetric experiments were performed using a CH Instruments Electrochemical Analyzer (Japan). A conventional three electrode system was employed with a platinum microcylinder as working electrode, platinum wire as an auxiliary electrode and Ag/AgCl as a reference electrode. The formal potentials ($E_{1/2}$) was taken as the average of the anodic ($E_{pa}$) and cathodic ($E_{pc}$) peak potentials.

2.4. Site specific binding of MT on HSA using marker ligands

The site specific binding of MT on HSA molecule has been studied with the know marker ligands bilirubin and diazepam, which specifically binds to site I and site II on HSA molecule. In this study, HSA was used as fluorescence probe and the changes in fluorescence intensity of HSA bound MT were monitored at 340 nm after excitation at 280 nm upon addition of varying amounts of marker ligands. The percent reduction in fluorescence intensity upon marker ligand binding was calculated considering the fluorescence intensity of MT-HSA complex as 100 percent and percent binding of MT at specific sites on protein.

2.5. Synchronous fluorescence analysis of MT treated HSA

To explore the structural change of HSA by the addition of MT, synchronous fluorescence analysis has been performed. In brief, the fixed concentration of HSA (3 $\mu$M) were titrated with varying molar ratios (0-10) of MT. The wavelength ranges of synchronous scanning were from 310 to 370 nm ($\Delta \lambda = 60$ nm) and 280 to 330 nm ($\Delta \lambda = 15$ nm) at 25 ± 0.1 °C.

2.6. Circular dichroism (CD) measurements

Effect of MT on the conformation of HSA were analysed by treating the fixed concentration of HSA (4.5 $\mu$M) with 0.1 and 0.2 $\mu$M of MT to get the MT/HSA molar ratio of 1:0.02 and 1:0.04. The untreated and MT treated samples were incubated for 2 h in white light at 37 °C. CD measurements were carried out on a Jasco spectropolarimeter, model J-815, Italy. The instrument was calibrated with d-10-camphorsulphonic acid. All
the CD measurements were performed at 25 °C with a thermostatically controlled cell holder attached to a NESLAB RTE-110 water bath (NESLAB Instruments, Inc. USA) with an accuracy of ± 0.1 °C. Spectra were collected at a scan speed of 100 nm per min with a response time of 1 sec. Each spectrum was the average of four scans. Far-UV CD spectra (200-250 nm) of the untreated and treated HSA were at a 1 mm path length cell. The protein samples for CD measurements were filtered through a Millipore filter (0.45 µM) to remove any suspended material. The results were expressed as mean residue ellipticity (MRE) in deg.cm².dmol⁻¹, which is defined as [MRE = θ obs (mdeg)/10 x n x l x Cp]. The θ obs represents the ellipticity in millidegree, n is the number of amino acid residues (585), l is the path length of the cell in cm and Cp is the mole fraction. The α-helical content of HSA was calculated from the MRE value at 222 nm using the equation % helix = [(MRE222 -2340)/730300] x 100 as described by Chen et al. (1972).

2.7. SDS-Polyacrylamide gel electrophoresis

SDS-polyacrylamide electrophoresis of untreated and MT treated HSA was carried out on 10 % (w/v) gel, according to the method of Laemmli (1970). In brief, HSA (25 µg) treated with increasing concentrations (1 to 5 mM) of MT was photoexcited for 2 h at 37 °C. The aliquots (6 µg each) of untreated control and treated protein were loaded on the gel and run at 3 mA per well for 3 h. The gel was stained with coomassie brilliant blue R-250 (0.25 % w/v) and destained with 5 % methanol and 7.5 % glacial acetic acid at room temperature.

2.8. MT-induced fragmentation of serum albumin

Fragmentation of HSA was assessed by measuring the TCA soluble amino groups according to the method of Moore and Stein (1954). A typical reaction mixture containing HSA (25 µM) with varying concentrations (200-1000 µM) of MT was exposed to white light for 2 h at 37 °C. The reaction was stopped with the addition of 100 µM EDTA followed by precipitation with 5 % (w/v) TCA. Supernatant was obtained after centrifugation at 2500 rpm for 30 minutes and the acid soluble amino group was quantitated using the calibration curve of glycine. Absorbance was read at 570 nm and plotted as a function of MT concentration. The carbonyl groups released from treated
serum albumin were also estimated using 2,4-dinitrophenylhydrazine reagent following the procedure of Lappin & Clark (1951).
3. Results

3.1. Fluorescence quenching of HSA upon MT interaction

The effect of MT on HSA was measured by monitoring the changes in the intrinsic fluorescence of serum albumin at different MT/HSA molar ratios. The fluorescence emission spectra of HSA alone and in presence of varying molar ratios (30:1) of MT/HSA was recorded in the in the range of 290-380 nm. The fluorescence intensities of protein decreased with increasing concentrations of MT. Figure 45 shows the fluorescence decay curves of the free HSA and HSA-MT complex. The titration curves exhibit a pronounced shift of about 30 nm in the emission maxima ($\lambda_{em}$) for HSA have been recorded. The relative fluorescence intensities of HSA at varying MT/HSA molar ratios were determined from the spectra of MT and plotted as a function of [MT]/[HSA] molar ratio (Figure 46). The binding isotherms and the corresponding Scatchard plots of $Q/[B] \times 10^6$ vs $Q$ for MT is shown in the inset of Figure 46. The binding isotherms exhibited a concentration dependent quenching of intrinsic fluorescence of protein. The total percent quench determined with different molar ratios of MT (2-30) were increased from 9.73 % to 64.42 % respectively. From the slope and intercept of the straight line obtained on the plot $Q/[B] \times 10^6$ vs $Q$, the binding constant ($K_d$) and binding capacity ($n$) of HSA towards MT were determined to be $4.5 \times 10^6$ l/mole$^{-1}$ and 26, respectively.

3.2. Cyclic voltammogram of MT-HSA solution

Figure 47 shows the cyclic voltammograms of MT in absence and presence of HSA. MT alone (Curve 1) exhibited a quasi reversible peak with an oxidation peak at -0.464 V and a reduction peak at -0.287 V. With the addition of equimolar concentration of HSA (1:1) the cathodic peak current ($i_{pc}$) of MT decreased from 3.210 to 2.886. The formal potential, $E'_{0}$ (or voltammetric $E_{1/2}$) taken as the average of $E_{pc}$ and $E_{pa}$ has been determined to be -0.376 with MT alone. Addition of HSA to MT solution slightly shifts the value of $E'_{0}$ towards less negative potential.

3.3. Site-specific binding of MT on HSA molecule

The competitive displacement of marker ligands specific for site-I (bilirubin) and site-II (diazepam) with MT-HSA complex demonstrated the site specificity of MT binding on
Figure 45. Fluorescence decay in the emission spectra of HSA in absence (uppermost curve) and presence of increasing amounts of MT. The spectra were obtained in 10 mM Tris-HCl buffer, pH 7.4 at 28 °C. The molar ratios of MT to HSA were: (top to bottom) 0.0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28 and 30, respectively. Fluorescence decay and shift in the emission spectra are indicated by down and horizontal arrows.
Figure 46. MT-HSA binding isotherm showing relationship between relative fluorescence and molar ratio in the range of (2-30). Inset shows the Scatchard plot drawn by fitting the fluorescence quenching titration data for determining MT-HSA binding parameters.
Figure 47. Cyclic voltammograms showing typical Nernstian behavior at a scan rate of 0.3 \text{Vs}^{-1}. Curve 1: MT (1mM); Curve 2: MT+HSA (1mM), respectively.
HSA molecule. The fluorescence emission spectra of MT-HSA complex at 340 nm reduced significantly with the addition of varying molar ratios of bilirubin, a shift of about 30 nm in the emission maxima has been observed with the addition of bilirubin (Figure 48). However, the addition of diazepam at the same molar ratios comparatively showed lesser reduction in the emission spectra of MT-HSA complex (Figure 49). Comparative data on the reduction in the initial fluorescence intensity of MT-HSA complex with the addition of bilirubin and diazepam are shown in Figure 50. The total % decline in the quenching effect of MT-HSA complex with the addition of bilirubin and diazepam in is presented Table 16.

3.4. Conformational changes in HSA upon interaction with MT

The synchronous fluorescence spectra present the information about the molecular microenvironment in the vicinity of the fluorophore functional groups. In the synchronous fluorescence of HSA, shift in position of maximum emission wavelength corresponds to the changes of polarity around the fluorophore of amino acid residues. The $\Delta \lambda$ values, (scanning intervals, $\Delta \lambda = \lambda_{emission} - \lambda_{excitation}$) stabilized at 15 or 60 nm, synchronous fluorescence of HSA gives the characteristic information of tyrosine residues and tryptophan residues, respectively (Miller, 1979). The effect of MT on HSA synchronous fluorescence spectroscopy is shown in Figure 51. As can be seen from the Figure 51 (A), the fluorescence of tyrosine residues was weak and the position of maximum emission wavelength had no change when $\Delta \lambda$ was 15 nm. However, the fluorescence of tryptophan residues was higher and the maximum emission wavelength show a moderate red shift when $\Delta \lambda$ was 60 nm (Figure 51, B).

3.5. Assessment of structural alterations in HSA upon MT treatment by CD analysis

CD spectra were also used to monitor MT-albumin interaction. Titration of HSA with increasing concentrations of MT at the molar ratios between (1:0.02 and 1:0.04) has been recorded in the far-UV CD spectral range of 200-250 nm (Figure 52). CD spectra of HSA exhibit two negative bands in the ultraviolet region at 209 and 222 nm characteristic for $\alpha$-helical structure of protein (He et al. 2005a). The reasonable explanation is that the negative peaks between 208, 209 and 222–223 nm are contributed to $n\rightarrow\pi^*$ transfer for
Figure 48. Fluorescence quenching of MT-HSA complex with the addition of increasing concentration of site I marker bilirubin. From top to bottom: HSA alone (3 μM), MT-HSA complex (10:1), bilirubin-HSA molar ratio 0.25, 0.5, 1.0, 1.5, 2.0, and 3.0, respectively.
Figure 49. Fluorescence quenching of MT-HSA complex with the addition of increasing concentration of site II marker diazepam. From top to bottom: HSA alone (3 μM), MT-HSA complex (10:1), diazepam-HSA molar ratio 0.25, 0.5, 1.0, 1.5, 2.0, and 3.0, respectively.
Figure 50. Decline in the initial fluorescence of HSA-MT (1:10) complex with the addition of increasing molar ratios of site I and site II markers (bilirubin and diazepam).
Table 16. Effect of marker ligands on the binding of MT to HSA

<table>
<thead>
<tr>
<th>MT-HSA molar ratio</th>
<th>Site I &amp; II Marker molar ratio</th>
<th>*Fluorescence quenching with marker ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bilirubin (Site I)</td>
</tr>
<tr>
<td>HSA alone</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HSA-MT (1:10)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HSA-MT (1:10)</td>
<td>0.25</td>
<td>41.56</td>
</tr>
<tr>
<td>HSA-MT (1:10)</td>
<td>0.50</td>
<td>54.18</td>
</tr>
<tr>
<td>HSA-MT (1:10)</td>
<td>1.0</td>
<td>69.3</td>
</tr>
<tr>
<td>HSA-MT (1:10)</td>
<td>1.5</td>
<td>77.54</td>
</tr>
<tr>
<td>HSA-MT (1:10)</td>
<td>2.0</td>
<td>79.73</td>
</tr>
<tr>
<td>HSA-MT (1:10)</td>
<td>3.0</td>
<td>83.09</td>
</tr>
</tbody>
</table>

*Calculated from the fluorescence intensity of HSA-MT complex in presence of marker ligands considering the fluorescence intensity of HSA-MT complex as 100.
Figure 51. Synchronous fluorescence spectrum of HSA in the absence and presence of MT. (A) Δλ = 15; (B) Δλ = 60. (1→11) HSA (3.0 μM), MT: 3,6,9,12,15,18,21,24, 27 and 30 μM, respectively.
Figure 52. CD spectra of HSA at different MT concentrations. (1) Free HSA, (2) MT 0.1 μM, (3) MT 0.2 μM.
the peptide bond of α-helix (Yang & Gao, 2002). A concentration dependent change in the ellipticity value has been recorded. The significant reductions in the MRE reflect the alterations in protein helicity. The percent changes in the α-helical content of the protein are shown in Table 17. At 0.2 μM, the loss in protein helicity was determined to be 8 % vis-à-vis 54.81 % α-helical content in native untreated HSA.

3.6. MT-induced fragmentation of HSA

MT-induced degradation of HSA has been analyzed on SDS-polyacrylamide gel (Figure 53 A). With increasing concentration, MT forms complex with HSA and induces fragmentation of the protein. Compared to the untreated HSA band in lane 2, a discernible reduction in the intensity of MT treated HSA bands were noticed. The densitometric analysis revealed a prominent loss (78.03 %) in parent band at the highest concentration of 5 mM of MT (Figure 53 B). The quantitative spectrophotometric assays demonstrated significant release of carbonyl and acid soluble amino groups from the HSA. The 2 h MT treatment to HSA at the varying concentrations of 200, 400, 600, 800 and 1000 μM has resulted in 72.2, 129.4, 222.4, 271.8 and 332.1 μM of carbonyl groups, respectively (Figure 54). An increase in the absorbance of blue color developed due to acid soluble amino groups was noticed up to 1000 μM MT at 570 nm. The absolute amount of acid soluble amino groups released from the MT (200-1000 μM) treated HSA were 1.1, 3.0, 4.1, 5.9 and 7.1 μM, respectively (Figure 55).
Table 17. Change in relative α-helicity of HSA upon interaction with MT.

<table>
<thead>
<tr>
<th>MT-HSA molar ratio</th>
<th>MRE$_{222}$ (deg.cm$^2$.dmol$^{-1}$)</th>
<th>α-helix</th>
<th>% perturbation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA (1:0)</td>
<td>18950</td>
<td>54.81</td>
<td>-</td>
</tr>
<tr>
<td>HSA-MT (1:0.02)</td>
<td>18142</td>
<td>52.15</td>
<td>5</td>
</tr>
<tr>
<td>HSA-MT (1:0.04)</td>
<td>17670</td>
<td>50.59</td>
<td>8</td>
</tr>
</tbody>
</table>
Figure 53. (A) SDS-PAGE of HSA showing loss of parent band with increasing concentrations of MT. Lane 1: 5 μg of standard protein marker; lane 2: untreated HSA 6 μg; lane 3: MT (1 mM) + HSA (6 μg); lane 4: MT (2 mM) + HSA (6 μg); lane 5: MT (3 mM) + HSA (6 μg); lane 6: MT (4 mM) + HSA (6 μg); lane 7: MT (5 mM) + HSA (6 μg), respectively. (B) Densitometric analysis of above bands showing the loss of parent HSA band.
Figure 54. Carbonyl group released from HSA treated with photosensitized MT for 2h. The data points represent the mean ± S.D. of three independent experiments done in duplicate.
Figure 55. Photosensitized MT induced protein fragmentation. HSA was treated with increasing concentration of MT for 2h and the amount of acid soluble amino group quantitated. The data represented are mean ± S.D. of three independent experiments done in duplicate.
4. Discussion

Fluorescence of HSA originates from tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) residues. In fact, the intrinsic fluorescence of HSA is mainly contributed by the tryptophan residue alone located at position 214 of the chain, because the phenylalanine residue has a very low quantum yield and the fluorescence of tyrosine is almost totally quenched when it is ionized or nearby an amino group, a carboxyl group or a tryptophan (Sulkowska, 2002). HSA emits strong intrinsic fluorescence at the excitation wavelength of 280 nm. This intrinsic fluorescence is very sensitive to its microenvironment, when local surroundings of HSA is altered even slightly, its intrinsic fluorescence would and factors such as protein conformational transition, biomolecule binding, and denaturation, etc. are in weakening the potential fluorescence (Yuan et al., 2007). The change of intrinsic fluorescence intensity of HSA is mainly due to the interaction of tryptophan residue with small molecules with HSA (Li et al., 2007). Thus, the intrinsic fluorescence of proteins is considered as an indicator of their structural and functional dynamics and helps in understanding the protein folding and association reactions (Wang et al., 2006).

In this study, the fluorescence intensity of HSA upon MT-HSA interaction undergoes proportionate reduction with the increasing concentrations of MT. Quenching of the HSA fluorescence could be due to change in microenvironment around the Trp 214 residue as has been suggested by several earlier workers (Trynda-Lemiesz et al., 1999; Yuan et al., 2007). The quenching of the tryptophan 214 fluorescence, clearly indicate that the conformation of the hydrophobic binding pocket in subdomain IIA is affected (Trynda-Lemiesz, 2004). Also, Zhang et al. (2007) have recently reported similar changes in HSA intrinsic fluorescence treatment with paraquat. Decay in the fluorescence intensity of HSA with the addition of MT also implies that the HSA molecule was quenched in S1 state. According to empirical rules for fluorescent spectra of proteins (Freifelder, 1976) the tryptophan residue in MT-HSA system is considered to be brought to a more hydrophilic environment as a result of the MT binding near the tryptophan residue or its proximity. The possible factors of fluorescence quenching were the alteration of HSA conformation and/or the binding of MT to HSA to result in
fluorescence energy transferring from tyrosine and/or tryptophan of HSA to MT. A prominent shift of emission maxima to a shorter wavelength on addition of MT to HSA indicate clearly the MT binding with tryptophan residue of HSA and induced conformational change in the native structure of protein as also reported earlier (He et al., 2005; Zhou et al., 2007). This effect was further validated by the synchronous fluorescence spectroscopy. Moderate shift of emission wavelength toward longer wavelength at λ60 and simultaneous decline in fluorescence reflects the fact that the microenvironment of the tryptophan residue was significantly affected by MT binding. It is reported that the maximum emission wavelength (λmax) at 330–332 nm indicates that tryptophan resides are located in the nonpolar region, are buried in a hydrophobic cavity in HSA. Also, the λmax at 350–352 nm shows that tryptophan residues are exposed to water, as the hydrophobic cavity in HSA is disagglomerated and the structure of HSA is compromised. Shift in the emission spectra in case of MT-HSA interaction suggests that MT bound to the hydrophobic cavity of HSA induce structural alterations in HSA as a consequence of increased polarity and reduced hydrophobicity (Wang et al., 2007a).

MT-HSA binding was further confirmed by simple and convenient electrochemical technique widely used to study the sub-layer structure and physiological functions of protein in the bioelectrochemistry (Hu et al., 2000). The results indicated that the addition of HSA to MT solution caused the decrease in the cathodic peak current (ipc). This could be explained in two possible ways, firstly the HSA interacts with MT to form a non-electrochemical complex, which blocks the electron transfer between the quasi reversible peaks of MT and electrode, and secondly the competitive adsorption of HSA at the glassy electrode surface (Zhao et al., 2007). Nonetheless, the cathodic peak current is closely related to the conformation changes of serum albumin, i.e. the microenvironment of electron transfer of protein at electrode. The native form of the protein has a specific binding site consisting of a hydrophobic patch close to a cationic group (Kelle y & McClements, 2003). To provide further information on the site-specific binding of MT, bilirubin and diazepam as a site I and site II markers, respectively have allowed to competitively bind with HSA. The maximum decrease in the fluorescence intensity (about 83.09 %) of HSA-MT complex in presence of bilirubin suggests that MT most
likely binds to the hydrophobic pockets at or near the site I within sub domain IIA of albumin molecule. The reduction is due to replacement of MT from site I which exhibits more affinity for bilirubin (Trynda-Lemiesz, 2004).

Conformational change in secondary structure of HSA has been ascertained using a sensitive circular dichroism upon interaction with MT lower molar ratios (1:0.02 and 1:0.04). The data distinctly exhibited decrease in the band intensities at 209 and 222 nm in the far-UV region without any significant shift of the peaks. This suggests a considerable change in the protein secondary structure, primarily due to reduction in α-helical content of treated protein. Furthermore, the results obtained from gel electrophoresis clearly show MT induced fragmentation of HSA. With the increasing concentration of MT, the parental band of HSA has disappeared, owing to hydrolysis producing a large number of indistinct random, fragments (Hawkins and Davies, 1999). The densitometric analysis of the band profile clearly shows the loss of the parent HSA band with increasing MT concentrations. Pesticides have also been reported for the interaction and complex formation with HSA (Purcell et al., 2001). The discernable protein fragmentation data of SDS PAGE has been further strengthened by the quantitative spectrophotometric data which indicate that the protein degradation may be due to peptide bond hydrolysis and/or chain scission at α-carbon position.

In conclusion, binding of MT with HSA and the consequent structural alterations in HSA were analyzed by fluorescence spectroscopy, cyclic voltammetry and CD spectroscopy. The fluorescence titration results showed that HSA possesses high binding affinity for MT with relatively higher binding number. MT selectively binds to the subdomain IIA on HSA, possibly by sharing the available lone pair of electrons between MT and HSA as substantiated by the CV data. The results of synchronous fluorescence and CD spectroscopy validated the MT-induced conformational change in HSA molecule. The conformational change leading to protein damage has been further confirmed by the spectrophotometer and gel electrophoresis data. Mechanism of MT induced HSA fragmentation is certainly due to chemical modification of critical amino acid residues. One possible modification could be the alkyl group transfer from MT to amino acid functional groups. The plausible theoretical model (Figures 56 and 57)
Figure 56. Proposed mechanism of MT-induced modification of tryptophan.
Figure 57. Proposed mechanism of MT-induced modification of tyrosine.
proposed to explain the MT induced protein-alkylation mechanism suggest that the amino group of tryptophan and tyrosine residues possessing free lone pair of electrons may act as a nucleophile. It attacks the ethyl cation and facilitate the release of (\(^\text{CH}_3\)) group from MT to side chain (\(^\text{NH}_3\)) group of tryptophan and tyrosine moieties on HSA. The protein containing dialkylated amino acids undergo fragmentation of the polypeptide chain. Thus the MT-HSA binding has demonstrated its toxicological implications. Till date no reports have been encountered on MT-HSA interaction. This study provides an important insight into the mechanism of interactions of a physiologically important protein HSA with pesticides and these measures are of particular interest in ecotoxicology and environmental risk assessment of MT.