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RESEARCH ARTICLE

IN VITRO THERMODYNAMIC AND SPECTROSCOPIC INVESTIGATION ON THE INTERACTION OF TRAMADOL HYDROCHLORIDE WITH BOVINE SERUM ALBUMIN MEASURED BY FLUORESCENCE QUENCHING METHOD

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ABSTRACT

To interpret the pharmacokinetics and pharmacodynamics properties of a drug molecule, plasma protein binding plays a substantial factor. The present study has been done to investigate the interaction of Tramadol hydrochloride (TRD) with bovine serum albumin (BSA) under physiological condition (pH 7.40) using UV absorption and fluorescence spectrophotometry at different temperatures (298K and 308K). Spectral methods are the most robust techniques for scrutinising the reactivity of chemical agents and biological systems since it permits nonintrusive measurements of the sample molecule in low concentration under identical physiological conditions. Quenching of BSA was also observed in presence of TRD by the fluorescence method. Quenching constants were determined at different temperatures (298K and 308K) using the Stern-Volmer equation. The thermodynamic parameters namely, enthalpy change (ΔH), entropy change (ΔS), and Gibb's free energy change (ΔG) were analysed based on Van't Hoff equation. It was found that with the increase of temperature, the value of Stern-Volmer constant increases in case of TRD. From this quenching mechanism, it was found that quenching of BSA-TRD system is static. Based on the thermodynamic parameters, hydrophobic interaction and hydrogen bonding were found to be involved in the formulation of complexes in BSA-TRD system. As Gibb's free energy change was negative, the interaction was a spontaneous process for the BSA-TRD system at both temperatures (298K and 308K). Binding constants (K) and the number of binding sites (n) were determined at different temperatures (298K and 308K). Based on the findings from the experiment regarding the interaction of BSA-TRD, the drug-protein binding mole ratio is 1:1 at temperatures 298K and 308K. The binding process is reversible and spontaneous.

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INTRODUCTION

The plasma protein binding profile of a drug molecule plays a vital role to comprehend the pharmacokinetics and pharmacodynamics properties of that molecule. It has arisen a great concern since it intensely effects drug distribution process and regulates the free fraction (Lazaro*et al.,* 2008). Most abundantly found proteins in blood are the serum albumins. They are engaged in various significant physiological functions. For example, they are mainly responsible for sustaining the blood pH and maintain the osmotic pressure (Hu *et al.,* 2006). However, the most important functions of serum albumins are the drug binding, transport and delivery of the drug through the bloodstream to their target organ (Tian *et al.,* 2004). Therefore, the investigation of such interaction of drug-protein complex has a great obligatory and inevitable significance (He and Carter, 1992). Bovine serum albumins and human serum albumins illustrate approximately 76% sequence homology and the 3D structure of BSA is considered to be similar with HAS (Shargel and Andrew, 2005). Since the presence of high-affinity binding sites for the formation of drug-protein complex and as the results obtained from the studies are consistent with HAS

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(Wise and Watters, 2011). In this investigation, bovine serum albumin (BSA) is carefully chosen as the target protein model. The drug- protein complex formed by the reversible and irreversible process (Shargel and Andrew, 2005). In the case of reversible processes, the drug-protein complexes formed through weaker chemical bonds, for example, hydrogen bonds or Van der Waals forces (Skoog, 2007). The reversible drug-protein complex formation has attained a great concern in pharmacokinetics. The formation of the irreversible drug-protein complex is due to the chemical activation of the drug candidates, which then tends to bind with the protein or macromolecule through stronger chemical bonds, for example, covalent bonds (Sultana *et al.,* 2013). Due to the formation of irreversible drug-protein complex formation or reactive chemical intermediates, it is chiefly responsible for certain types of toxicities, for example, chemical carcinogenesis which may occur as a result of prolong use. If protein binding of a drug molecule is declined, the free concentration of that drug will increase eventually. Therefore, more drugs will be available to interact with the receptor site. This will produce an intense pharmacological effect (Zhang *et al.,* 2008). The duration of action of a drug molecule is also influenced by the protein binding. Although researchers have performed various experiments to elucidate the structure, the properties of serum albumin and their possible way of interaction with small molecules such as dyes, drugs and toxic chemicals using the fluorescence method (Ran *et al.,* 2007; Wang *et al.,* 2007; Yuan *et al.,*1998; Zhang *et al.,* 2013). But there has been no report on the interaction of Tramadol with protein albumin. In this regards, the present work has been performed to obtain a detail and insightful information of protein binding of Tramadol hydrochloride with BSA by considering the interactions using mainly UV-visible and fluorescence spectroscopy. The aim is to optimise the use of Tramadol as a predictive, preventive, safe and personalised medicine.

MATERIALS AND METHODS

For the study of the reactivity of chemical and biological systems, spectroscopic methods are the most influential tool. As it permits a nonintrusive measurement of substances at low concentrations, it is widely used for the measurement under physiological conditions (Grond and Sablotzki, 2004; Li *et al.,* 2007). Here fluorescence spectroscopy and UV spectroscopy were used to perform the study. 100 ml of a 0.1M solution of Tramadol hydrochloride was prepared as the stock solution by taking 2.9983g of Tramadol hydrochloride (Mol. Wt. 299.836) in a 100 ml volumetric flask and dissolving in nano-pure water. Bovine serum albumin (BSA) was used as the target protein model. Bovine serum albumin is a fatty acid-free, fraction V, and 96-98% pure protein which was purchased from the Sigma-Aldrich Chemical CO, USA. A solution of BSA was prepared at a concentration of 2.5×10^{-6} M by measuring 41.875 mg of protein correctly and dissolving in a 250 ml volumetric flask with nano-pure water. The process was done carefully in order to ensure that there was no foam formation. The protein solution was preserved at 4^{o} C until further use. Since BSA is a fluorescence active molecule in this system, the intensities at the excitation wavelength of 280 nm and 293 nm for BSA were taken. The fluorescence spectra were measured at different temperatures (298 K and 308 K).

RESULTS AND DISCUSSION

Analysis of fluorescence quenching mechanism: Fluorescence quenching is the reduction of the quantum yield of fluorescence from a fluorophore. When a fluorophore is substantially induced with quencher molecule by a variety of molecular interaction (i.e. excited state reactions, energy transfer, molecular rearrangements and ground-state complex formation) quenching of fluorescence is evident (Silva *et al.,* 2004; Cui *et al.,* 2007). In order to ensure the quenching mechanism, the fluorescence data are usually evaluated by Stern-Volmer equation (Bhattacharyya *et al*., 1990):

$$F_0/F = 1 + K_{SV}(Q)$$

Here $F_0$and F are the fluorescence intensities of BSA in the absence and presence of quencher, respectively. (Q) denotes the concentration of quencher which is TRD in this case, and K_{SV} is the Stern-Volmer quenching constant, which signposts the strength of the interaction between the TRD and BSA. $K_{SV}$is the slope of the plot of F_0/F against (TRD) based on the fluorescence data at different temperatures.

Effect of TRD on the fluorescence spectra of BSA at 298 K

The fluorescence spectra of BSA was measured in the absence and presence of various concentration of TRD at the excitation wavelength of 280 nm and 290nm.

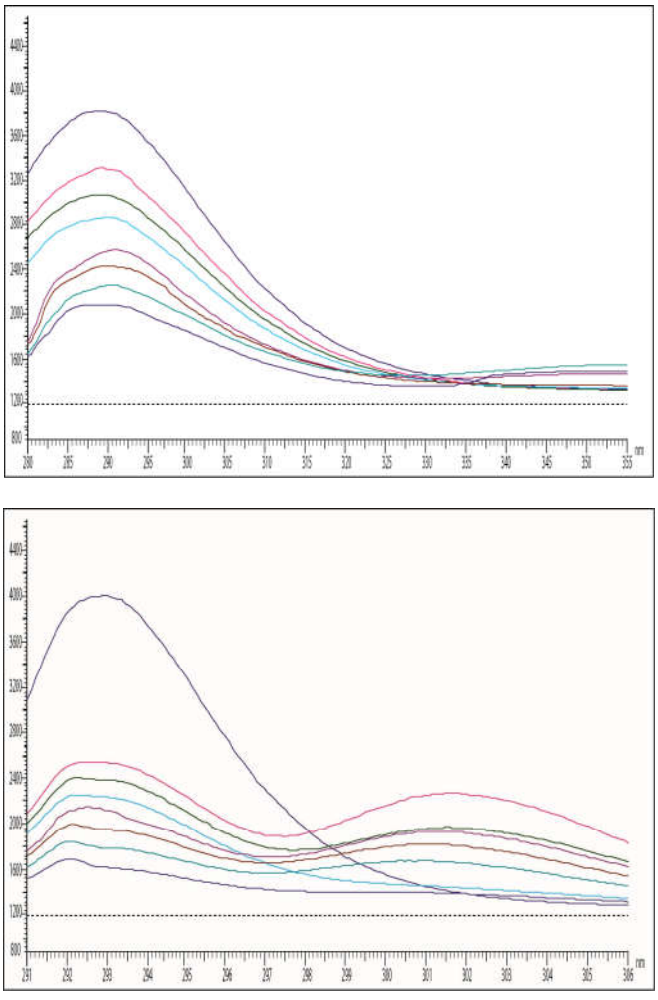

Figure 1. Fluorescence titration curve of BSA in the presence of TRD at the excitation wavelength of 280 nm and 293 nm at 298 K

The fluorescence spectra of BSA demonstrate a broad band with the absorption maximum at 283.4 nm. Fluorescence quenching is observed for BSA in the presence of different TRD concentration. Quenching spectrum is a hint of a sturdy

interaction and energy transfer occurred between TRD and BSA at 298 K. If a ligand is adequately close to tryptophan and tyrosine fluorescent residues, a decrease in intrinsic fluorescence intensity can be detected. To resolve whether both the tryptophan and tyrosine residues are engaged in the interaction with TRD, the fluorescence intensities of BSA at the excitation wavelength of 280 nm and 293 nm was measured in the presence of TRD. When the excitation wavelength of 280 nm is used, the fluorescence of albumin comes from both tryptophan and tyrosine residues, whereas when the excitation wavelength of 293 nm is used, it only excites the tryptophan residues (Parisa *et al.,* 2013).

temperatures, listed in Table 3.2. The Stern-Volmer quenching constant decreases with increasing temperature for static quenching while for dynamic quenching the reverse effect (Steinhardt *et al.,* 1971). From Table 3.2, it is clear that the probable quenching mechanism of the TRD- BSA binding reaction is not initiated by dynamic quenching but by static quenching resulted from the complex formation.

Thermodynamic parameters and nature of the binding forces

The forces involve the interaction between quencher and fluorescence active molecule may be included as the

Table 1. Data for interaction of TRD with BSA at 298K

	F_0	F	F/F_0	(TRD)/(BSA)		F_0	F	F/F_0	(TRD)/(BSA)
	3999	3950	1.0124	1		4245	4105	1.0341	1
	3999	3545	1.1281	2		4245	4085	1.0392	2
	3999	3758	1.0641	4		4245	3971	1.0690	4
280 nm	3999	3733	1.0713	6	293 nm	4245	3904	1.0873	6
	3999	3603	1.1099	8		4245	3657	1.1608	8
	3999	3557	1.1243	12		4245	3622	1.1720	12
	3999	3390	1.1796	16		4245	3478	1.2205	16

The plot F/F_0 against (TRD)/ (BSA) is presented in Figure 2, which indicates that in the presence of TRD, the fluorescence of BSA at the excitation wavelength of 280 nm obviously diverges from that the excitation wavelength of 293 nm. This substantial differences between the fluorescence quenching of serum albumin illustrate that there are alterations in the participating fluorophores of BSA at different wavelengths. At 280 nm fluorescence of BSA comes from both tryptophan and tyrosine residues, and at 293 nm fluorescence of BSA only involves tryptophan residue (Steinhardt *et al.,* 1971).

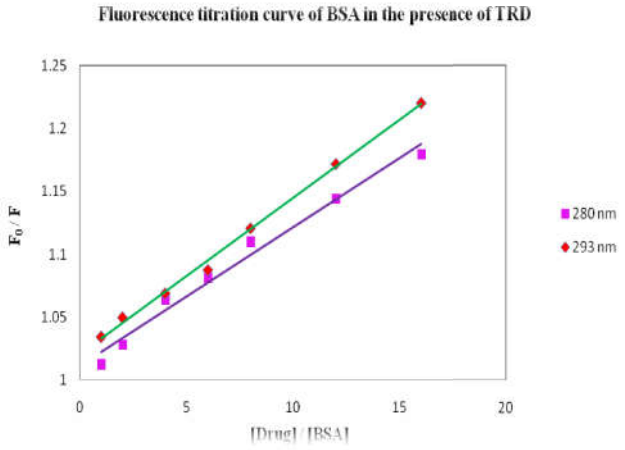

Figure 2. The plot F/F0 against (TRD)/ (BSA)

Effect of TRD on the fluorescence spectra of BSA at 308 K

The fluorescence spectra of BSA with varying concentration of TRD at the excitation wavelength of 280 nm at 308K. The fluorescence spectra of BSA show a broadband with an absorption maximum at 312.5 nm. The quenching of fluorescence is observed for BSA in the presence of different TRD concentration. This quenching spectrum is a hint of a sturdy interaction and energy transfer between TRD and BSA at 308 K. The plot displays that in the above-mentioned concentrations, the results are in accordance with the Stern-Volmer equation. The plots are linear and Stern-Volmer quenching constants are obtained from the slopes at various

hydrophobic force, electrostatic interactions, Vander Waals interactions, hydrogen bonds, etc. (Liu Xuyang *et al.,* 2007). Thermodynamic parameters namely the change of enthalpy, the entropy and Gibb's free energy are anticipated for revealing the interaction between the TRD and BSA.

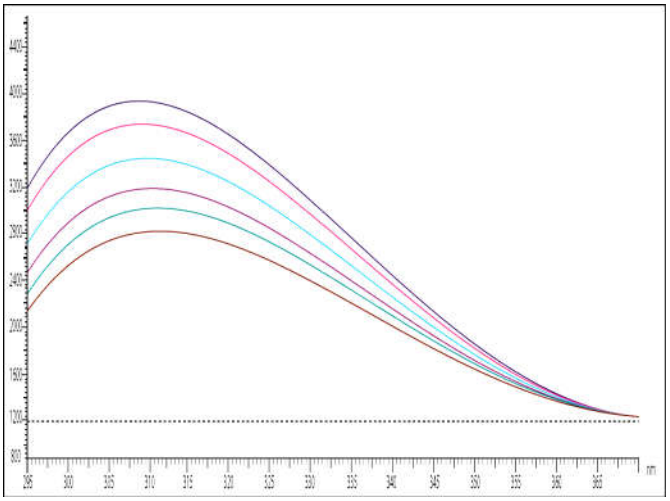

Figure 3. Fluorescence titration curve of BSA in the presence of TRD at the excitation wavelength of 280 nm at 308 K

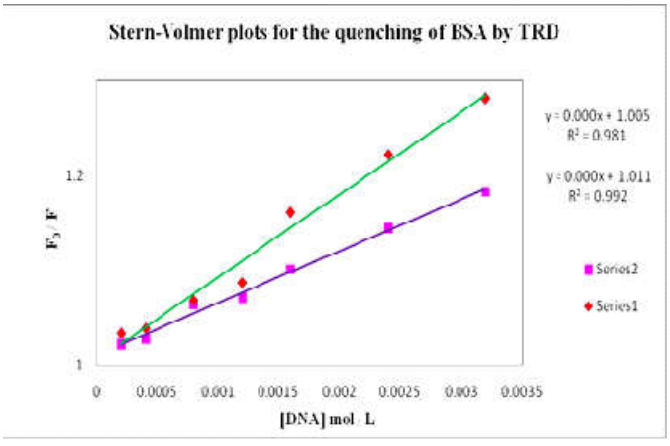

Figure 4. The Stern-Volmer plots for the quenching of BSA by TRD at 298K and 308K

Table 2. Stern-Volmer quenching constant K_{SV} of the system of TRD- BSA at 280nm (R =Correlation co-efficient)

T (K)	K_{SV} (L mol^{-1})	R
298	0.0009	0.9923
308	0.0005	0.9818

The main reasons for carrying out the thermodynamic study of BSA-TRD interactions is to scrutinise the factors responsible for overall binding affinity and specificity of the drug molecule for the protein (Papadopoulou *et al.,* 2005). The thermodynamic parameters can be assessed from the Van't Hoff equation:

$$\ln K_a = - (\Delta H/RT) + (\Delta S/R)$$

Here ΔH, ΔS denotes as the change of enthalpy and the entropy, correspondingly; constants K_a are equivalent to the Stern-Volmer quenching constants K_{SV} at the corresponding temperature (Haq, 2002), R is the gas constant. If the temperature does not differ so meaningfully, then the change of enthalpy (ΔH) can be regarded as a constant (Sun *et al.,* 2006). The change of enthalpy (ΔH) and the entropy (ΔS) can be assessed from the slope and intercept of the fitted curve of $\ln K_{SV}$ against $1/T$, correspondingly.

From the subsequent equation Gibb's free energy (ΔG) can be assessed:

$$\Delta G = \Delta H - T\Delta S$$

The temperature changes affect these binding forces as follows:

- When ΔH and ΔS are positive, it indicates a typical hydrophobic interaction (He *et al.,* 2010).
- When ΔH and $\Delta S$are negative, it is an indication of Vander Waals and hydrogen bonding in low dielectric media (He *et al.,* 2010).
- Negative ΔH might have a role in electrostatic interactions, ΔH is assumed to be very little or almost zero (He *et al.,* 2010).
- For the spontaneous binding of biomolecules and ligand. ΔG is always negative (Aki and Yamamoto, 1989).

Table 3. Data of Van't Hoff plot for BSA-TRD system at 280 nm at two different temperatures

K_{SV} (L mol^{-1})	ln K_{SV}	T	1/T
0.0009	-7.601	298	0.00336
0.0005	-7.013	308	0.00324

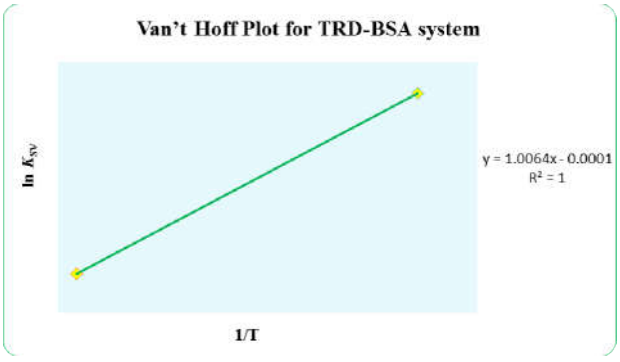

Figure 5. The Van't Hoff Plot for BSA-TRD system at 280 nm at two different temperatures

Table 4. Thermodynamic parameters of the system of BSA-TRD at 280nm

T(K)	ΔH (KJ/mol)	ΔS (J/mol)	ΔG (KJ/mol)
298	0.0009	1.0056	-299.66
308	0.0005	1.0119	-311.66

From the Table 3.4, the change of free energy (ΔG) is negative, the change of enthalpy (ΔH) and the entropy (ΔS) are positive. Different sign and magnitude of the thermodynamic parameters gives the impression of different interaction forces (Ross and Subramanian, 1981) such as the positive value of ΔS andΔH is considered as the evidence of hydrophobic interaction from the point of view of H_2O molecule structure while negative ΔH and ΔS values considered as the evidence of hydrogen bonds, Van der Waals interactions and protonation additionalassociation. Electrostatic interactions are characterised by positive ΔS and when ΔH is almost zero(Aki and Yamamoto, 1989). The negative sign for ΔG reveals that the binding process is spontaneous (Aki and Yamamoto, 1989). Thus it can be settled that hydrogen bonding and Van der Waals interactions are present in the BSA-TRD binding.

Binding constant and binding points

Once small molecule resides independently to a set of equivalent sites on a macromolecule, the equilibrium constant between free and bound molecule can be attained from the subsequent equation (Ross and Subramanian, 1981):

$$\log ((F_0 - F)/ F) = \log K + n \log (Q)$$

Here, K and n denote the binding constant to a site and the number of binding per molecule, correspondingly. From the values of intercept and slope of the plot of log $((F_0\text{-}F)/F)$ versus log (TRD), the values of K and n are intended, correspondingly.

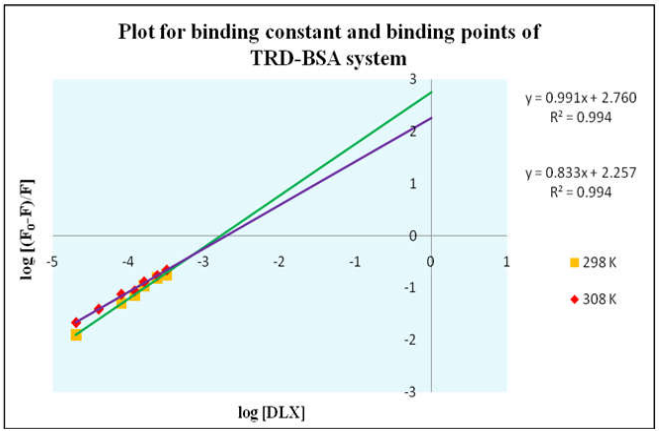

Figure 6. Plot for the determination of binding constant and binding points of BSA-TRD system at 298K and 308 K

From the table 3.6, it is evident thatthe values of K and n, at 280 nm are different at different temperatures (298K and 308K), which was obtained from the intercept and slope, separately. It was detected that the binding constant decreases with the increase in temperature, resulting in the reduction of the stability of the BSA-TRD complex, which is a further proof that the fluorescence quenching mechanism of BSA-TRD system is static. The values of n remained constant at different temperatures. From the experimental data, it is evident that the binding mole ratio for the BSA-TRD system is 1: 1.

Table 5. Data regarding binding constant and binding points for BSA-TRD system at 298 K and 308K

	F_0	F	log ((F_0-F)/F)	(TRD) mol/L	log (TRD)		F_0	F	log ((F_0-F)/F)	(TRD) mol/L	log (TRD)
	3999	3950	-1.906	20×10^{-6}	-4.699		4245	4105	-1.467	20×10^{-6}	-4.699
	3999	3545	-0.892	40×10^{-6}	-4.397		4245	4085	-1.407	40×10^{-6}	-4.397
	3999	3758	-1.192	80×10^{-6}	-4.096		4245	3971	-1.161	80×10^{-6}	-4.096
298K	3999	3733	-1.147	120×10^{-6}	-3.920	308K	4245	3904	-1.058	120×10^{-6}	-3.920
	3999	3603	-0.958	160×10^{-6}	-3.795		4245	3657	-0.793	160×10^{-6}	-3.795
	3999	3557	-0.905	240×10^{-6}	-3.619		4245	3622	-0.76	240×10^{-6}	-3.619
	3999	3390	-0.745	320×10^{-6}	-3.494		4245	3478	-0.65	320×10^{-6}	-3.494

Table 6. Binding constant and binding points of the system of TRD- BSA at 280nm

T (K)	*K* (L/mol)	*n*
298	2. 7602	0.8336
308	2. 2576	0.9911

CONCLUSION

Analyses of the interactions of Tramadol hydrochloride (TRD) with BSA under the physiological buffer (pH 7.4) were carried out by fluorescence spectroscopic technique. The fluorescence quenching of BSA was also observed in presence of TRD. The experimental results indicated that tyrosine residues also participate with tryptophan residues in the interactions of TRD with BSA at 280 nm excitation wavelength.From Stern-Volmer constant value at different temperature (298 and 308K), the quenching of fluorescence of BSA initiated by TRD was found to be static. In order to determine the factors responsible for the overall binding affinity and specificity of the drugs, thermodynamic parameters namely enthalpy change (ΔH), entropy change (ΔS), and Gibb's free energy (ΔG) for BSA-TRD system was measured on the basis of Van't Hoff equation. The observation demonstrates the existence of hydrogen bonding and hydrophobic interaction in BSA-TRD complex formation which indicates reversible binding. The interaction process was spontaneous and the mole ratio of BSA-TRD is 1:1 (which indicated that 1 mole of TRD binds with 1 mole of BSA). The binding process is reversible and spontaneous.The experimental consequences bear a great impact in pharmacy, pharmacology and biochemistry, and are expected to provide important insight into the interaction of the drug molecule with protein albumin. From the protein binding study, accurate calculation of different pharmacokinetic parameters of Tramadol is possible. Thus, the study will be helpful in designing actual dosage regimen and finding out new drugs with lower side effects. So from the interaction study of BSA-TRD, future study can be done besides having an analgesic effect. This study will help to discover some new kind of pharmacological properties of Tramadol hydrochloride and to design a predictive, preventive, safe and personalised medicine.

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