Original Research Article

In Vitro analysis of the interaction between ketorolac tromethamine and bovine serum albumin using fluorescence spectroscopy

ABSTRACT

Objectives: To evaluate the *in vitro* mutual interaction of ketorolac tromethamine with bovine serum albumin (BSA) using fluorescence spectroscopy under different conditions.

Material and methods: Different concentrations of ketorolac were mixed with 20 µM bovine serum albumin solution at pH 7.4 and stirred for 2 min at 298 K & 308 K temperatures. Finally different ketorolac-BSA complex at the excitation wavelength 280 nm & 293 nm was measured by the fluorescence spectrophotometer.

Results: In the experimental work, it was found that ketorolac is responsible for fluorescence quenching of BSA molecule where tryptophan and tyrosine both amino acid participated in the molecular interactions between BSA and ketorolac at excited state. Stern-Volmer equation is used to calculate fluorescence quenching constant. The thermodynamic parameters such as Gibb's free energy (ΔG), enthalpy change (ΔH), and entropy change (ΔS) at different temperatures were studied by using Van't Hoff equation. The values of ΔG, ΔH and ΔS at 298K were -33.25 KJ/mol, 17.093 KJ/mol and 168.94 J/mol for ketorolac. The binding process for ketorolac had been found to be spontaneous, exothermic and entropy driven as indicated by thermodynamic analysis and hydrophobic forces playing a major role in the ketorolac-BSA association.

Conclusions: The interaction of ketorolac with BSA was successfully explored using a fluorescence spectroscopic technique.

Key words: Ketorolac tromethamine, bovine serum albumin, fluorescence quenching, Thermodynamic parameters.

INTRODUCTION

One of the most important physiological functions of albumins is their ability to carry drugs, endogenous and exogenous substances. Various experiments were done previously for characterizing the binding capacity and binding sites of albumins. The fluorescence spectral changes observed on the binding of drug with bovine serum albumin (BSA) are important tools for the investigations of drug binding sites, conformational changes and for the characterization of substrate to ligand binding [1]. Serum albumin

being the major transporters binding protein for the drugs and other physiological substances, it is considered as a *in vitro* model for studying drug–protein interaction.

Protein binding of a drug is a limiting factor for drug effect. The type and nature of protein binding depend on the physicochemical properties of the drug molecules, their concentration, pH of the medium and also on the concentration and number of available binding sites of the plasma proteins [2]. Serum albumins are the most abundant proteins in blood as it binds with the drug and forms a drug-protein complex which strongly influences drug distribution and the free fraction, which is available to the target [3].Therefore the investigation of such molecules with respect to albumin binding is of imperative and fundamental importance. The interaction between drug molecule and protein may result in the formation of stable and reversible drug-protein complex having important effect on the distribution, metabolism and excretion of drug [4]. Human and bovine serum albumins have approximately 76% homology. 3D structure of BSA is quite similar to that of HSA. In this experiment BSA is selected as our protein model because of its low cost, ready availability and medical importance .All studies are consistent with the fact that human and bovine serum albumins are homologous proteins [5]. Spectroscopic methods are powerful tool for the study of the reactivity of chemical and biological systems since it allows measurements of substance in low concentration under physiological conditions, and there are several studies of albumin induced by drugs or other bioactive small molecules using spectral methods [6].

In this work, fluorescence spectroscopic method is used to investigate the interaction of Ketorolac with BSA molecule to evaluate the binding mechanism of ketorolac to BSA such as participating aminoacid residues, fluorescence quenching rate constant, thermodynamic parameters, binding constant and also the number of binding sites. Ketorolac tromethamine is a non-steroidal anti-inflammatory drug (NSAID) that exhibits analgesic activity. Ketorolac inhibits the synthesis of prostaglandins through inhibition of the cyclooxygenase enzyme system. Ketorolac tablets are available in the market for the treatment of fever and inflammation or swelling and relieve pain. It is also used to treat pain, swelling and stiffness associated with arthritis.

MATERIALS AND METHODS

Materials:

Ketorolac (KT) was obtained from Incepta Pharmaceuticals Ltd. Bangladesh. BSA (fatty acid free, fraction V, 96-98%) was purchased from Sigma Chemical CO, USA. All other chemicals were of analytical reagent grade and purchased from local supplier. Doubly distilled water was used throughout the work.

Apparatus:

Steady-state fluorescence measurements were carried out on a Hitachi F-7000 fluorescence spectrophotometer (Hitachi, Japan) equipped with 1.0 cm quartz cells. For different temperature a thermostat bath (Unitronic Orbital, P- Spectra, Spain) was used.

Spectroscopic measurement:

The fluorescence spectra of drug were performed at different temperatures (298º K and 308º K). The widths of both entrance and exit slit were set to 5 nm. Most of the fluorescence emission spectra in the range of 320- 460 nm were recorded at excitation wavelength of 280 nm and 293 nm, respectively, in the same experimental conditions. Ketorolac has no fluorescence in the range of 320- 460 nm. The mixture solution of BSA and ketorolac must be hatched at least 10 min before the spectrum measurements.

Principle of fluorescence quenching:

Fluorescence quenching is the decrease of the quantum yield of fluorescence from a fluorophore induced by a variety of molecular interaction with quencher molecule [5]. The fluorescence quenching data are usually analyzed by Stern- Volmer equation [6]:

$F_o/F = 1 + K_{SV}[Q]$……..(1)

Where, Fo and F are the fluorescence intensity of fluorophore in the absence and presence of quencher, respectively. [Q]is the concentration of quencher and K_{SV} is Stern-Volmer quenching constant, which indicates the strength of the interaction. K_{SV} is the slope of the plot of F_o/F against [Q] based on the fluorescence data at different temperatures.

RESULTS AND DISCUSSION

The interaction of Ketorolac with BSA:

Phenylalanine, tyrosine and tryptophan are three amino acids with intrinsic fluorescence properties, but quantum yields of tyrosine and tryptophan are high enough to give a good fluorescence signal. At the excitation wavelength of 280 nm, both tyrosine and tryptophan amino acids are exited. But at 293 nm selectively excite tryptophan residue

In order to determine whether both tyrosine and tryptophan residues are involved in the interaction of ketorolac and BSA, the fluorescence spectrum of BSA excited at 293 nm and 280 nm in the presence of ketorolac is compared. Fluorescence of albumin comes from both tryptophan and tyrosine residues when 280 nm excitation wave length is used,, whereas 293 nm selectively excite tryptophan residue [6].

The plots F/Fo against [Ketorolac]/[BSA] at excitation wavelength 280 nm and 293 nm are measured to determine the interaction of drug and BSA. Here, F_o is the fluorescence intensity of BSA, F is the fluorescence intensity of BSA in presence of drug.

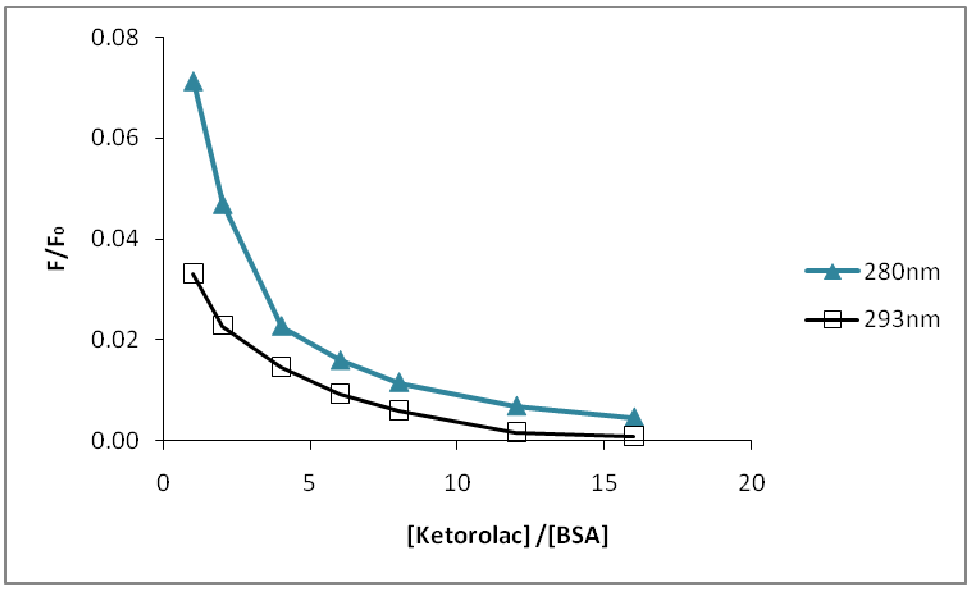
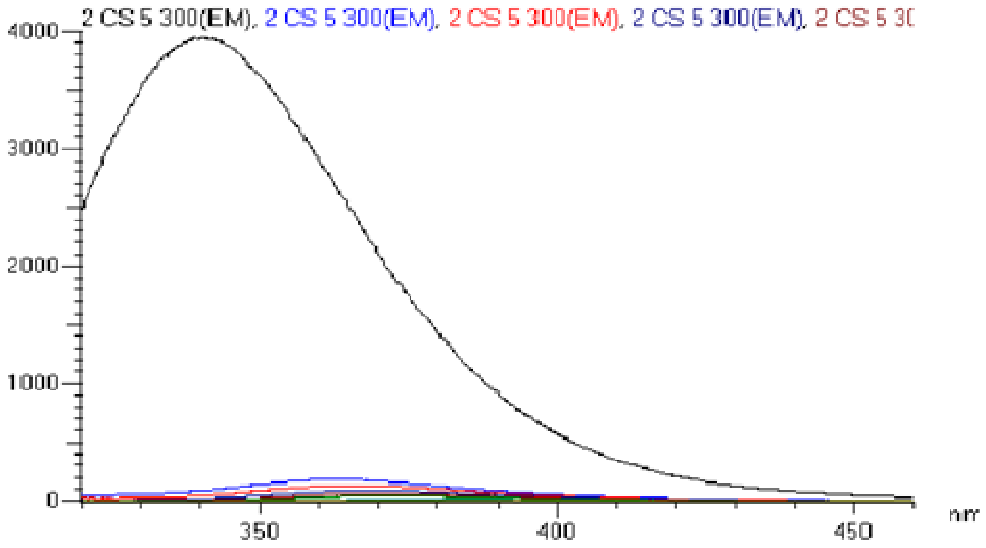


Fig. 1: The plots of F/Fo against [Ketorolac]/[BSA] concentrations at 280 nm and 293 nm

From the plots F/F_o versus [Ketorolac]/[BSA] were shown in the **fig. 1**, we concluded that the fluorescence of BSA excited at 280 nm and at 293 nm in the presence of ketorolac is obviously different. This significant difference between quenching of serum albumin fluorescence indicated that both tyrosine and tryptophan aminoacids participated in the molecular interactions of BSA and ketorolac at excited state.

Fluorescence spectra of BSA in presence of ketorolac :

The fluorescence spectra of BSA with varying concentration of ketorolac at the excitation wavelength of 280 nm and 293 nm, are shown in the **fig. 2** and **fig. 3** respectively. From the spectra it was observed that fluorescence quenching of BSA in the presence of different concentration of ketorolac was occurred at 298K.



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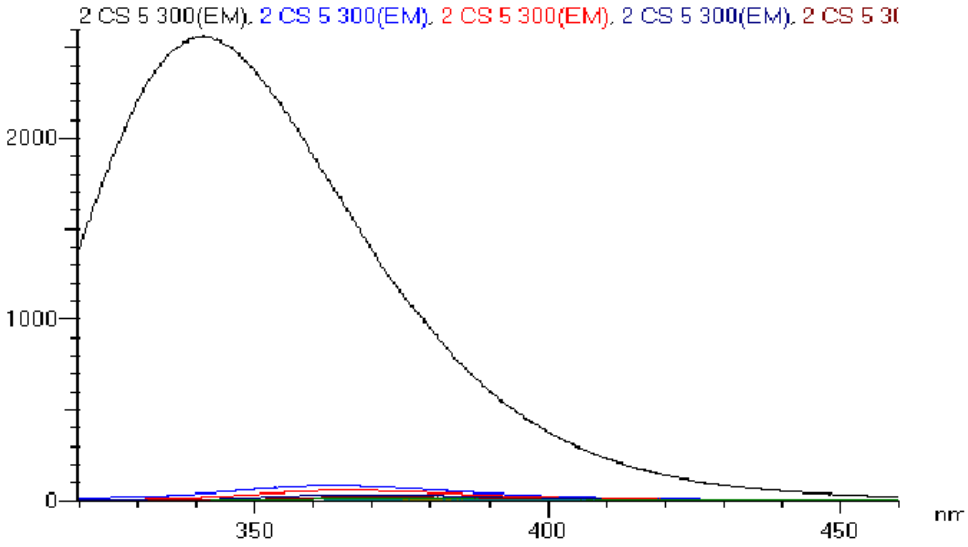
107 **Fig. 2:** Fluorescence emission spectrum of BSA in the presence of ketorolac at 280nm and 298K



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109 **Fig. 3**: Fluorescence emission spectrum of BSA in the presence of ketorolac at 293 nm and 298K)

110 **Analysis of fluorescence quenching mechanism:**

111 Fluorescence quenching is the decrease in fluorophore fluorescence intensity induced by a variety of
112 molecular interaction with any other molecule [5]. Molecular interactions can result in quenching including
113 molecular rearrangements, excited state reactions, collisional quenching, ground state complex formation
114 and so on. The quenching mechanisms are usually classified as either dynamic quenching or static
115 quenching [6]. Dynamic quenching refers to the collision of the quencher and fluorophore during the
116 excitation while static quenching refers to formation of complex between quencher and the fluorophore
117 [7].

The fluorescence quenching constant are calculated by Stern-Volmer equation [5]:

$$F_o/F = 1 + K_{SV}[Q]$$

K_{SV} was calculated from the slope of the plot of F_o/F versus [Ketorolac] based on the fluorescence data at different temperatures (298ºK and 308ºK).

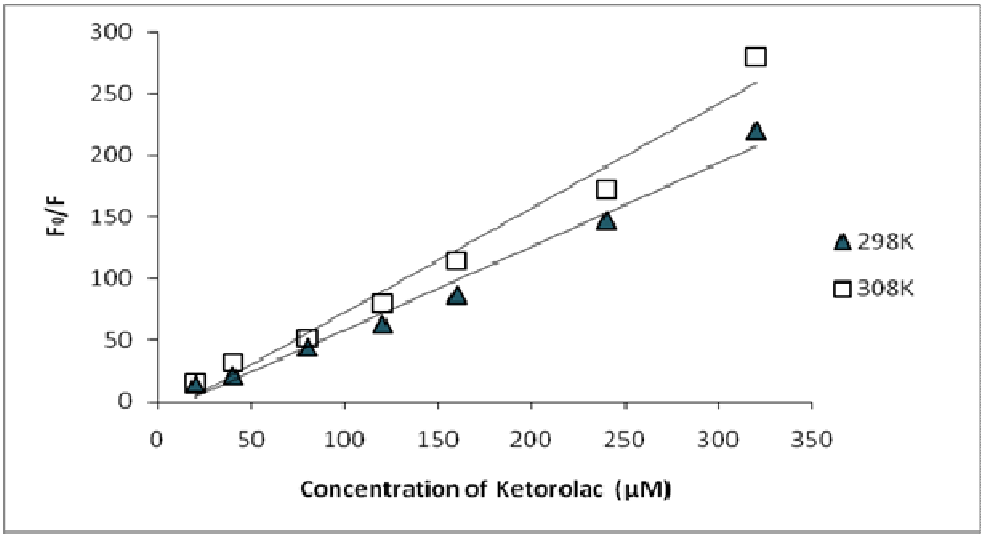

Fig. 4: The Stern-Volmer plots for the quenching of BSA by ketorolac at 298K and 308K.

The quenching of BSA fluorescence by the ketorolac at different temperatures is displayed in **Fig.4**. The plots were linear and showed that the results were in good agreement with the Stern-Volmer equation within the experimental concentrations. Stern-Volmer quenching constants were obtained from the slopes at various temperatures and those are listed in **Table 1**. For dynamic quenching,the Stern-Volmer quenching constant increases with increase in temperature while for the static quenching reverse effect is observed [8]. It was clear from Table 1 that the probable quenching mechanism for the ketorolac-BSA binding reaction was due to dynamic quenching.

Table 1: Stern-Volmer quenching constant (K_{SV}) of ketorolac-BSA system (R= Correlation co-efficient)

T (K)	K_{SV} **(L mol^{-1})**	**R**
298	6.77 x10^5	0.984
308	8.47 x10^5	0.977

Thermodynamic parameters and nature of the binding forces:

Hydrophobic force, electrostatic interaction, van der Waals interaction, hydrogen bond, etc are involved in the interaction forces between quencher and fluorescence active molecule[9]. The thermodynamic parameters like ΔS, ΔH *and* ΔG were calculated in order to determine the interaction between the ketorolac and BSA. The thermodynamic parameters can be determined from the Van't Hoff equation:

$$\ln K_a = -(\frac{\Delta H}{RT}) + (\frac{\Delta S}{R})$$

Where,ΔS = Entropy change; ΔH = Enthalpy change; K_a = analogous to the Stern-Volmer quenching constants K_{SV} at the corresponding temperature [3]; R = Gas constant.

ΔH and ΔS can be determined from the slope and intercept of the curve of $\ln K_{SV}$ versus $1/T$, respectively.

The Gibbs free energy, ΔG can be estimated from the following relationship:

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

Hydrogen bonds, van der Waals forces, hydrophobic forces and electrostatic forces are involved for reversible drug-protein binding, binding forces like [10]. The Van't Hoff Plot for ketorolac-BSA system was shown on **Fig. 5.**

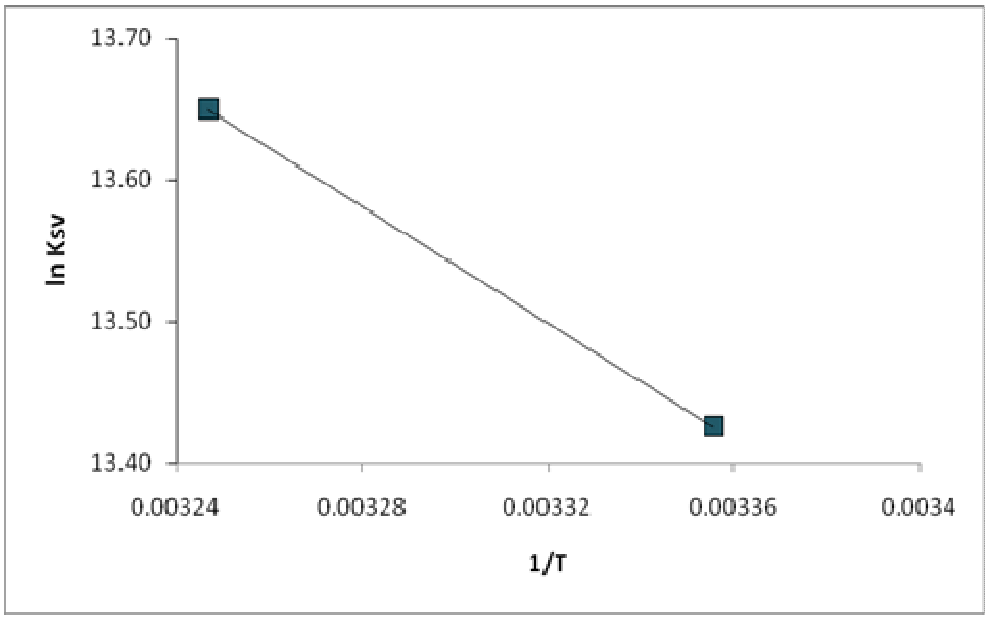

Fig. 5: The Van't Hoff Plot for ketorolac-BSA system; [BSA] = 20 µM

Table 2: Thermodynamic parameters of ketorolac-BSA binding

T (K)	ΔH (KJ/mol)	ΔS (J/mol)	ΔG (KJ/mol)
298	17.093	168.94	-33.25

As shown in **Table 2,** the enthalpy change (ΔH) and the entropy change (ΔS) were positive, and the free energy change (ΔG) was negative. The negative value of ΔG indicated that binding of ketorolac to BSA was spontaneous. According to the rules summarized by Ross and Subramanian[11], for drug - protein interaction, the positive ΔS and ΔH values of interaction of ketorolac and BSA indicated that binding is mainly entropy driven and enthalpy is unfavorable for it, the hydrophobic forces playing a major role in the ketorolac-BSA system.

Binding constant and binding sites for ketorolac:

When small molecules like drug bind to different sites on a protein, the equilibrium between free and bound drug molecules is given by the following equation [12]:

$$\log\left\{\frac{(F_0 - F)}{F}\right\} = \log K + n\log[Q]$$

Where,

K=the binding constant to a site of albumin protein

n= the number of binding site for drug per albumin.

From the values of intercept and slope of the plot of log {(Fo - F)/ F} versus log [Ketorolac], respectively the values of K and n were calculated shown in the **Fig. 6**.

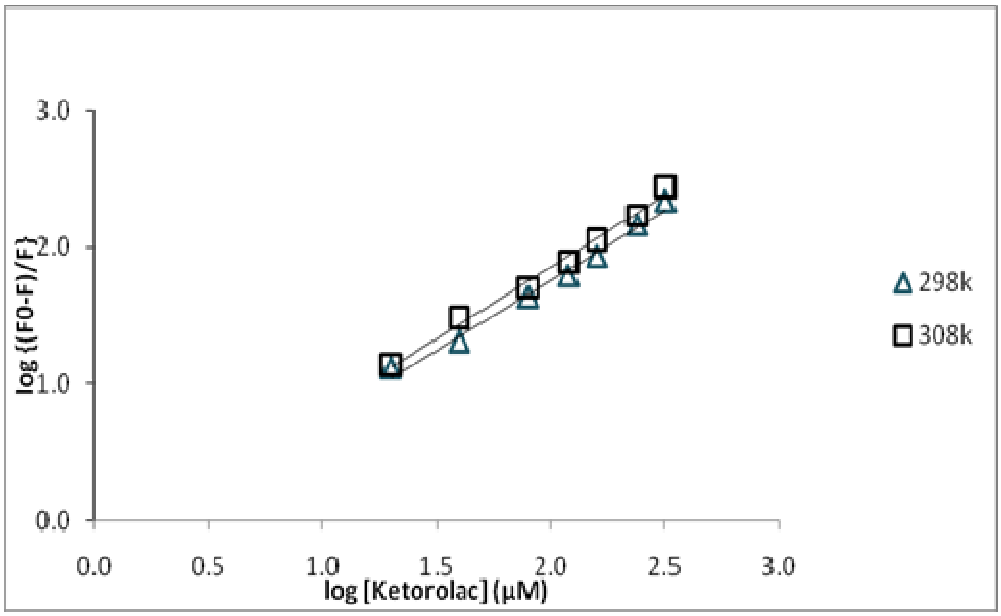

Fig. 6: Plot for binding constant and binding sites of ketorolac at 298K and 308K

Table 3: Binding constant and binding sites of ketorolac-BSA system

T (K**)**	***K* (μM^{-1})**	***n***
298	0.522	1.020
308	0.603	1.038

Table 3 contained the values of *K* and *n*, at different temperatures, which were obtained from the intercept and slope of **Fig.6**. It was observed that the binding constant increases with the increase in temperature, resulting in increasing the collision of ketorolac-BSA system. The obtained values for *n* were found to be ≈ 1 indicate that only a single ketorolac molecule binds with BSA and temperature has almost no effects on bindings sites of drug on BSA.

CONCLUSION

The pharmacological activity of a drug depends on protein binding. A drug activity may increase or decrease by any kind of change in drug-protein interaction. In this experiment, fluorescence spectroscopic method has been used to observe the interactions of ketorolac with BSA. The result indicates that tyrosine and tryptophan both amino acids participate in the interactions between ketorolac and BSA at excited state. From the fluorescence quenching data we found that interactions of BSA and ketorolac are a result of dynamic quenching. The Stern-Volmer equation was used to calculate fluorescence quenching constants and van't Hoff equation to measure the thermodynamic parameters

ΔG, ΔH, and ΔS at different temperatures. The binding process for ketorolac has been found spontaneous, exothermic and entropy driven as indicated by thermodynamic analysis and hydrophobic forces playing a major role in the ketorolac-BSA association. This interaction of ketorolac with albumin give us an idea about the consequences of dose increment of this drug [13].Ketorolac is a NSAID and it needs to take as prolong therapy in acute to chronic pain management of the patients. So, drug-protein interaction study of such kind can be helpful for safe dosing regimen to minimize the toxicity of the drug and for better therapeutic outcome.

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