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# **Insight into the interaction of Inhaled Corticosteroids (ICS) with human serum albumin: a spectroscopic-based study**

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## **KEYWORDS**

Human Serum Albumin

Inhaled corticosteroids

Fluorescence spectroscopy

FRET

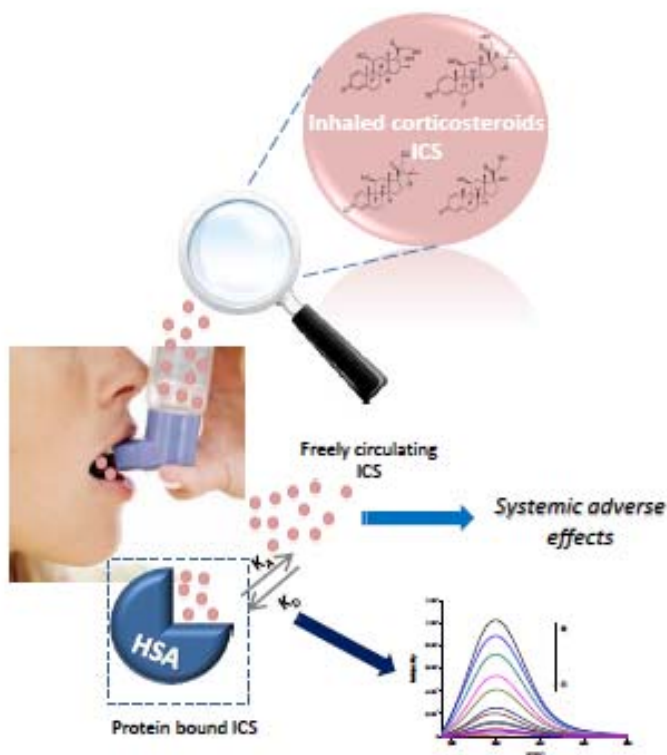
Binding

## **1. INTRODUCTION**

The study of protein–drug interactions plays an important role in pharmacokinetics and pharmacodynamics of drugs. They influence the distribution and elimination speed; only non-binding drug is able to spread and reach the target producing a biological response. One of the most important factors affecting the distribution and the free, active concentration of many administered drugs is binding affinity for human serum albumin (HSA). Drug binding to HSA increases drug half-life and lowers the free drug concentration in blood, which makes it extremely important for clinical care. In early drug discovery, the plasma protein binding is important in order to evaluate drug dosing needs and clearance from the body.

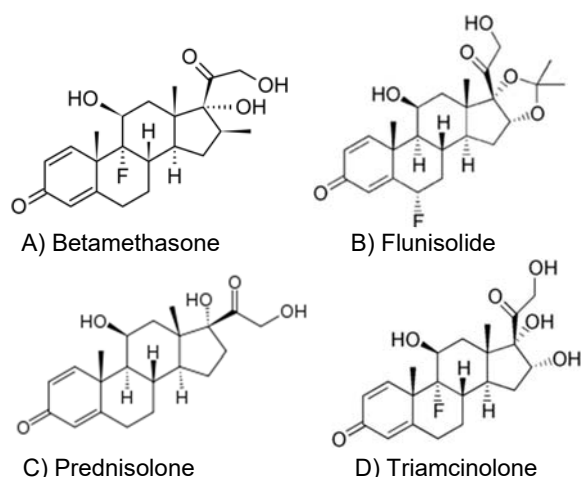
From a pharmaceutical point of view, very interesting is the interaction between Inhaled Corticosteroids (ICSs) and HSA. Corticosteroids are the most potent and effective anti-inflammatory agents in many respiratory chronic diseases. In this case, the preferred way of administration of a corticosteroid is inhalation; this way permits to deliver the drug directly to the lung, where it acts locally in order to minimise the systemic sides-effects, compared to oral or parenteral administration. Clinical studies have shown that ICSs significantly reduce airway hyperresponsiveness, effectively prevent acute exacerbations, improve lung function and decrease symptoms severity [1]. Corticosteroids are involved in different physiological processes, in particular they alter the production of inflammatory mediators in the airways, such as macrophages, eosinophils, lymphocytes, mast cells and dendritic cells [2].

It is well known that the safety and efficacy profile of an ICS is influenced by the pharmacokinetic properties and associated pharmacodynamic effects of the drug [3, 4]. Freely circulating, protein unbound, active ICS can cause systemic adverse effects. In fact, the freely circulating ICS could bind to nonpulmonary glucocorticoids receptors and can cause adverse effects such as a reduction in the function of the hypothalamic-pituitary-adrenal (HPA) axis and growth impairment [5]. Extensive protein binding can, therefore, be viewed as a way to temporarily remove an ICS that is available to the tissues from the systemic circulation, thereby reducing the potential for development of adverse effects [5- 7]. Among other pharmacologic properties, high plasma protein binding is a desirable property for any ICS, since this reduces the potential for systemic side effects [8]. Therefore, a detailed investigation of drug–protein interaction assumes significance for thorough understanding of the pharmacokinetic behavior of corticosteroids and for the design of analogues with effective pharmacological properties (Fig1).



**Fig.1** The interaction of ICSs with albumin plays an important role in governing systemic side effects.

The purpose of this study was to evaluate the extent of protein binding of different ICSs, like betamethasone (A), flunisolide (B), prednisolone (C) and triamcinolone (D) (Fig.2) in order to develop a rapid spectroscopic method to study the interaction and eventually compare the affinity of ICS with HSA. In our knowledge the interaction between ICS and HSA has never been investigated by spectroscopic techniques.



**Fig.2** Structures of the studied ICSs.

HSA is the most abundant drug carrier protein, with a well known primary structure. Its tertiary structure has been determined by X-ray crystallography [9]. It has an important role in maintaining the colloidal osmotic pressure in blood and in the transport of exogenous and endogenous substances, including fatty acids, amino acids, steroids, bilirubin and drugs [10, 11]. Backbone of protein consists in a single polypeptide chain of 585 amino acid residues, that form three homologous domains (I, II, and III), stabilized by 17 disulfide bridges, which are formed due to the 34 cysteines present in the molecule; each domain contains two subdomains (A and B), respectively constituted by 6 and 4  $\alpha$ -helices [12- 14]. Crystallographic studies have revealed that HSA has binding sites for aromatic and heterocyclic ligands within two hydrophobic pockets: in subdomains IIA (Sudlow's site I: warfarin-binding site) and IIIA (Sudlow's site II: indole/benzodiazepine site). Both hydrophobic and electrostatic interactions play a major role in controlling the affinity towards drug binding for sites I and II; for site I, mainly hydrophobic interactions are dominant, while for site II, a combination of hydrophobic, hydrogen bonding and electrostatic interactions play a crucial role. The tryptophan residue (Trp 214) of HSA is in subdomain IIA (site I) and plays a crucial role in spectrophotometric studies [10, 11]. When a ligand binds to one domain can induce a distinct conformational changes on the other domain, as both subdomains share a common interface. For this reason, the binding of a

drug to serum albumin may change considerably binding abilities of HSA towards other molecules [15].

In this study, UV-Vis and fluorescence spectroscopy were used to elucidate the mode of binding and probable structural alterations of HSA upon drug binding. The binding constant and the nature of binding forces were determined. Lastly, the thermodynamic and Förster's parameters associated with the binding process were also calculated. All the data obtained could clarify the type of interaction that can occur between ICS and HSA and could be fundamental to understand if and how the structural features of the drugs could modulate this interaction. Moreover it should pave the comprehension of the bioavailability of corticosteroids, justifying the major use as inhaled administration and may facilitate the interpretation of absorption and distribution process of corticosteroids.

## **2. MATERIALS AND METHODS**

### **2.1 Materials**

Albumin from human serum (HSA) lyophilized powder,  $\geq 97\%$  (agarose gel electrophoresis) was purchased from Sigma Aldrich. To prepare the stock solution (100  $\mu\text{M}$ ), HSA was dissolved in PBS (phosphate buffer solution, pH 7.4; 2 mM).

Betamethasone ( $\geq 98\%$ ), flunisolide ( $\geq 97\%$ ), prednisolone ( $\geq 99\%$ ) and triamcinolone were all purchased from Sigma Aldrich; the stock solutions (3 mM) were prepared by dissolving drugs in a solution 1:1 of ethanol 96% and PBS.

### **2.2 Apparatus**

All fluorescence spectra were recorded with a Horiba Jobin Yvon Fluorolog3 TCSPC spectrofluorophotometer with 1.0 cm quartz cells. UV-Vis spectra were recorded on a UH5300 Hitachi spectrophotometer. The pH measurements were made with a Eutech Instruments pH2700.

### **2.3 Experimental conditions**

HSA 5  $\mu\text{M}$  was titrated by successive additions of drugs solutions at different concentrations; fluorescence quenching spectra were measured in the range of 300-500 nm upon excitation at 280 nm. The excitation and emission slits were respectively 6 nm and 10 nm. The fluorescence spectra were performed at three different temperatures (296 K, 303 K, 310 K). To reach protein saturation, it is necessary to use a range from 50  $\mu\text{M}$  to 500  $\mu\text{M}$  for betamethasone and from 50  $\mu\text{M}$  to 700  $\mu\text{M}$  for flunisolide, prednisolone and triamcinolone.

UV-Vis measurements were recorded in the range of 200-400 nm. UV-Vis absorption spectra were recorded at room temperatures, by using different concentrations of drugs (betamethasone, prednisolone, triamcinolone= 2.0, 4.0, 6.0, 8.0, 10.0  $\mu\text{M}$ ; flunisolide= 5.0, 10.0, 15.0, 20.0, 25.0  $\mu\text{M}$ ).

## **3. RESULTS AND DISCUSSION**

### **3.1 UV-Vis spectroscopy**

UV absorption spectroscopy is one of the techniques used to explore the structural changes of protein and to investigate protein-ligand complex formation [16]. HSA has two main absorption bands, and one of them is located at 280 nm, which is the absorption band of the tryptophan (Trp 214) [17, 18].

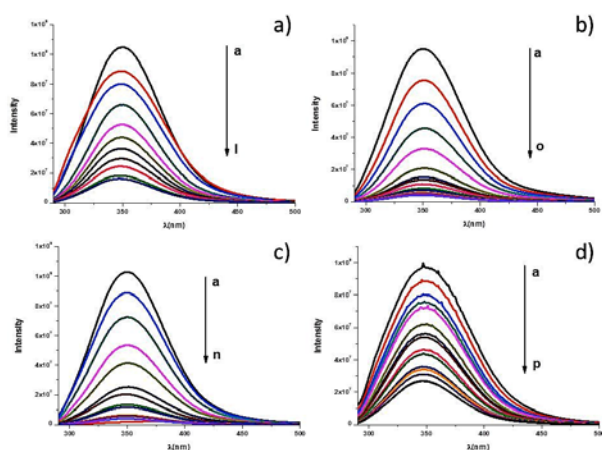
The absorption spectra of the protein at room temperature in absence and in presence of different concentrations of drugs betamethasone, prednisolone, triamcinolone= 2.0, 4.0, 6.0, 8.0, 10.0  $\mu\text{M}$ ; flunisolide= 5.0, 10.0, 15.0, 20.0, 25.0  $\mu\text{M}$  were recorded and shown in Fig. S1. As can be seen, for



every sample, the absorption intensity of HSA at around 280 nm increased with the addition of increasing concentrations of drugs. Moreover, the absorption spectrum of protein-drug complex is different from that of albumin and drugs alone. The maximum peak position of HSA-drugs complex was slightly shifted towards lower wavelength region. These results confirmed that every studied drug could bind the protein.

### 3.2 Fluorescence quenching mechanism

The fluorescence spectrum of albumin was recorded in absence and in presence of drugs at different concentrations (Fig. 3 shows spectra at room temperature).



**Fig.3** Fluorescence spectra of HSA–drugs interaction ( $T = 296$  K). A) Fluorescence spectra of HSA–betamethasone: [HSA] black line; a-l [betamethasone] = (50.0- 500.0)  $\mu$ M. B) Fluorescence spectra of HSA–flunisolide: [HSA] Black line; a-o [flunisolide] = (50.0- 700.0)  $\mu$ M. C) Fluorescence spectra of HSA–prednisolone: [HSA] = black line; a-n [prednisolone] = (50.0- 700.0)  $\mu$ M. D) Fluorescence spectra of HSA–triamcinolone: [HSA] = black line; a-p [triamcinolone] = (50.0- 700.0)  $\mu$ M.

HSA shows a typical strong fluorescence emission peak at 350 nm, which does not shift in the presence of the drugs. Every analysed molecule causes a concentration dependent quenching of the intrinsic fluorescence of protein that decreases gradually with the increase of drug's concentration. The quenching of protein fluorescence by drugs was due to the formation of a protein-drug complex. This means that the microenvironment of HSA was changed during the binding interaction. In order

to obtain thermodynamic parameters, binding studies were performed at three different temperatures (296 K, 303 K and 310 K) and the obtained steady-state maximum fluorescence intensity was recorded.

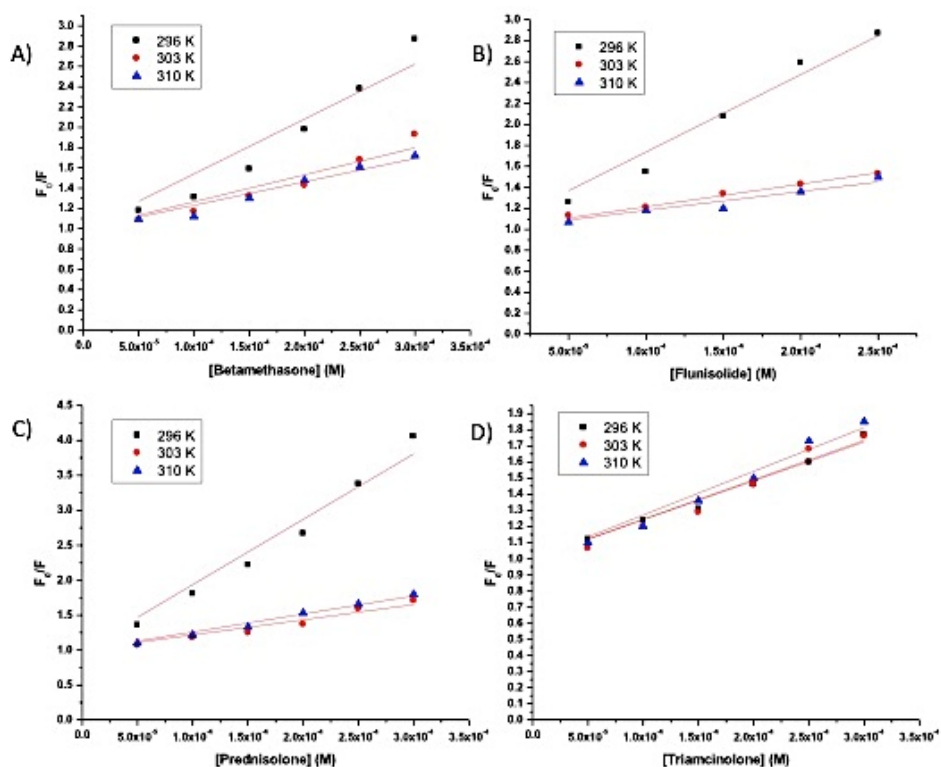
Fluorescence quenching data were treated by different methods, as reported in the following paragraphs, to evaluate the equilibrium association ( $K_A$ ) and dissociation ( $K_D$ ) constants.

### 3.2.1 Stern-Volmer equation

First of all, fluorescence quenching of albumin was analysed by Stern-Volmer equation (1) [19]:

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

where  $F_0$  is the fluorescence intensities in the absence of quencher,  $F$  are the steady-state fluorescence intensities in the presence of the quencher and  $[Q]$  is the concentration of the quencher.  $K_{SV}$  is the Stern-Volmer quenching constant and describes a collisional quenching of fluorescence. Quenching data are presented as plots of  $F_0/F$  vs.  $[Q]$ , yielding an intercept of one on the y-axis and a slope equal to  $K_{SV}$ . In Fig. 4 Stern-Volmer plots at different temperatures are reported.



**Fig.4** The Stern Volmer plots of the fluorescence quenching of HSA by drugs at different temperatures; A) betamethasone; B) flunisolide; C) prednisolone; D) triamcinolone.

A linear Stern-Volmer plot, however, does not define the quenching mechanism. In order to distinguish dynamic from static quenching, the dependence of the interaction of a drug, described by  $K_{SV}$ , from temperature has been proposed. The  $K_{SV}$  values decrease with an increase in temperature for static quenching and the reverse effect will be observed for dynamic quenching [19].

As shown in Table 1, the  $K_{SV}$  of the complex protein-drug A, B, C decreases with increasing temperature.

296 K	303 K	310 K
$K_{SV}(M^{-1})$	$K_{SV}(M^{-1})$	$K_{SV}(M^{-1})$

<b>A</b>	5408	2657	2312
<b>B</b>	7385	2160	1807
<b>C</b>	9358	2169	2582
<b>D</b>	2415	2447	2709

Table 1 The quenching constants of HSA and drugs: A) betamethasone, B) flunisolide, C) prednisolone and D) triamcinolone at different temperatures.

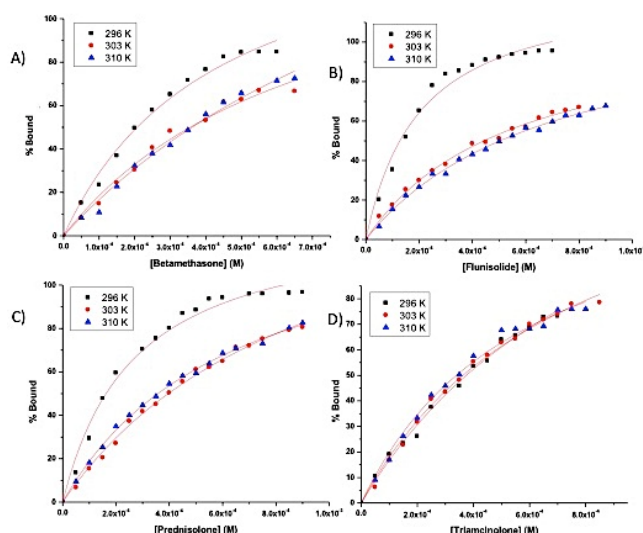
This indicates that probably a static quenching interaction between protein and drug occurs [20]. The  $K_{sv}$  of protein-triamcinolone complex is similar with negligible variations by changing the temperature, but it is possible to observe a slight increase also in this case, so probably a static quenching interaction between protein and drug may also occur.

### 3.2.2 Non-linear least squares

Non-linear least squares fit procedure is a simple method to analyse fluorescence data at different temperatures, [21] based on Eq. 2:

$$y = \frac{B_{max}[Q]}{K_D + [Q]} \quad (2)$$

where  $[Q]$  is the drug concentration,  $y$  is the specific binding derived by measuring fluorescence intensity,  $B_{max}$  is the maximum amount of the complex protein/drug formed at saturation and  $K_D$  is the equilibrium dissociation constant. In Fig. 5 the binding curves obtained are reported; the percentage of bound HSA, i.e.  $y$ , derived from the fluorescence intensity emission maximum, is plotted against the drug concentration.



**Fig.5** The binding curves of HSA–drugs complex at different temperatures. A) betamethasone; B) flunisolide; C) prednisolone; D) triamcinolone.

The corresponding  $K_D$  and  $K_A$  (which are reciprocals of each other) at different temperatures are shown in Table 2.

	296 K		303 K		310 K	
	$K_A$	$K_D$	$K_A$	$K_D$	$K_A$	$K_D$
	( $M^{-1}$ )	( $\cdot 10^{-4} M$ )	( $M^{-1}$ )	( $\cdot 10^{-4} M$ )	( $M^{-1}$ )	( $\cdot 10^{-4} M$ )
	Non linear fit					
<b>A</b>	2288	4.37	1414	7.07	840	11.9
<b>B</b>	4926	2.03	1789	5.59	1414	7.08
<b>C</b>	3731	2.68	1063	9.41	1553	6.44
<b>D</b>	1020	9.80	1323	7.56	1698	5.89

Table 2 Values of the equilibrium dissociation and association constants of HSA- drugs complex at different temperatures, obtained by a non linear fit equation.

The binding constant calculated for HSA-triamcinolone complex suggests the lower affinity of this drug for the protein than the other tested drugs, as reported in literature [8]. The  $K_A$  of protein-triamcinolone complex is similar with negligible variations by changing the temperature, but it is

possible to observe a slight increase. Also in this case, affinity seems to be higher for flunisolide. By increasing temperature, for A, B and C analysed complex, the value of association constant decreases.

### 3.3 Binding parameters

By double logarithm regression curve (shown in equation 3) [22], it is possible to obtain the number of binding sites ( $n$ ). This equation describes the relationship between fluorescence intensity and the quencher concentration:

$$\log \frac{(F_0 - F)}{F} = \log K_A + n \log [Q] \quad (3)$$

where  $F_0$  is the fluorescence intensity of the protein alone,  $F$  is the fluorescence intensity after the addition of the quencher and  $[Q]$  is the quencher/ drug concentration. The slope of the line is the  $n$  value. If the value of  $n$  is equal to 1, it means that a strong binding exists between the protein and the drugs [22].

The number of binding sites is easily calculated: for HSA–betamethasone complex is 1.50 (296 K), 1.30 (303 K), 1.50 (310 K), for HSA–flunisolide is 1.30 (296 K), 0.92 (303 K), 1.10 (310 K), for HSA–prednisolone complex is 1.30 (296 K), 1.40 (303 K), 1.30 (310 K) and for HSA–triamcinolone is 1.30 (296 K), 1.40 (303 K) 1.30 (310 K). Almost all values are approximately equals to 1, indicating that there is one independent binding site on HSA for every analysed drug [23]

### 3.4 Site Marker Competitive Binding Experiments.

In order to further investigate drugs binding site on HSA, competitive binding tests have been carried out. Warfarin and ibuprofen are two markers specific for binding to HSA that bind to sites I and II, respectively. Therefore, to determine the location of corticosteroids binding site on HSA, the competitive displacement experiments were studied using warfarin as a characteristic marker for site I and ibuprofen for site II [24]. In the site marker competitive experiment, warfarin or ibuprofen were

gradually added to the solution of HSA-corticosteroids complex and then fluorescence intensity of the system was recorded. As shown in Figure S2, with addition of warfarin in the HSA solution, the fluorescence intensity was slightly higher than that of without warfarin (and including the red shift). Then, after adding the warfarin into the solution of HSA-corticosteroids complex, the fluorescence intensity of HSA solution decreased gradually, and the intensity was much lower than that without warfarin, displaying that the binding of the corticosteroids to HSA was affected after adding warfarin. On the contrary, in the presence of ibuprofen, the fluorescence intensity of the HSA-ibuprofen complex almost had no difference from that recorded without ibuprofen under the same conditions (Figure S3), which indicated that site II marker did not prevent the binding of corticosteroids in its usual binding location. These results suggest that corticosteroids compete with warfarin for binding to HSA to site I [24].

### 3.5 Thermodynamic parameters

The interaction forces between small molecules and macromolecules include four binding modes: H-bonding, Van der Waals, electrostatics and hydrophobic interactions [25]. The model of interaction between drug and the protein could be obtained, according to the data of enthalpy ( $\Delta H$ ) and entropy change ( $\Delta S$ ) [26]: (1)  $\Delta H > 0$  and  $\Delta S > 0$ , hydrophobic forces; (2)  $\Delta H < 0$  and  $\Delta S < 0$ , van der Waals interactions and hydrogen bonds; (3)  $\Delta H < 0$  and  $\Delta S > 0$ , electrostatic interactions. The thermodynamic parameters, enthalpy and entropy of the HSA-drugs complex reaction are important to confirm binding modes. The temperature-dependence of the binding constant was analysed at 296 K, 303 K, and 310 K and thermodynamic parameters were calculated from the following Van't Hoff equations [19]:

$$\ln K_A = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (4)$$

$$\Delta G = -RT \ln K_A \quad (5)$$

$$\Delta S = \frac{\Delta H - \Delta G}{T} \quad (6)$$

where  $K_A$  is the binding constant,  $R$  is the gas constant and  $T$  is the experimental temperature. The values of  $\Delta H$  and  $\Delta S$  obtained for the binding sites are shown in Table S4. The negative sign for  $\Delta G$  means that the binding process is spontaneous for every studied interaction [23].

From Table S4 it can be seen that for HSA-betamethasone, HSA-flunisolide and HSA-prednisolone complexes both  $\Delta H$  and  $\Delta S$  have a negative value. This indicates that van der Waals interactions and hydrogen bonds may play a major role in the binding. Conversely, in the formation of HSA-triamcinolone complex an exothermic reaction occurs, characterized by a negative  $\Delta H$  value and a positive  $\Delta S$  value. From the point of view of water structure, a positive  $\Delta S$  value is frequently taken as a typical evidence for hydrophobic interaction. Furthermore, specific electrostatic interactions between ionic species in aqueous solution are characterized by a positive  $\Delta S$  value and a negative  $\Delta H$  value [27]. In order to evaluate the thermodynamic parameters of HSA-triamcinolone complex, it is not possible to account of a single intermolecular force model. As described in literature for another complex (dexamethasone- HSA) [28], the binding, in this case, might involve hydrophobic interaction strongly, as evidenced by the positive values of  $\Delta S$ , but electrostatic interaction can also not be excluded.

### 3.6 Energy transfer

FRET (Fluorescence Resonance Energy Transfer) is a simple method to measure the distance between the acceptor (ligand) and the donor (tryptophan residues in the protein) [29]. According to Förster's non-radiative energy transfer theory, energy efficiency  $E$ , critical energy-transfer distance  $R_0$  ( $E= 50\%$ ), the energy donor and the energy acceptor distance  $r$  and the overlap integral between the fluorescence emission spectrum of donor and the absorption spectrum of the acceptor  $J$  can be



calculated by the following equations [30]:

$$E = 1 - \left(\frac{F}{F_0}\right) = \frac{R_0^6}{R_0^6 + r^6} \quad (7)$$

$$R_0^6 = 8,79 \times 10^{-5} [\kappa^2 n^4 \varphi J(\lambda)] \quad (8)$$

$$J = \frac{\sum F(\lambda) \varepsilon(\lambda) \lambda^4 \Delta\lambda}{\sum F(\lambda) \Delta\lambda} \quad (9)$$

where  $k^2$  is the orientation factor,  $\varphi$  is the fluorescence quantum yield of the donor,  $n$  is the refractive index of the medium,  $F(\lambda)$  is the fluorescence intensity of the donor at wavelength  $\lambda$  and  $\varepsilon(\lambda)$  is the molar absorption coefficient of the acceptor at wavelength  $\lambda$ . In this case,  $k^2=2/3$ ,  $n= 1.336$  and  $\varphi = 0.118$  [16].

The overlaps of the emission spectra of the protein and the absorption spectra of drugs at room temperature were obtained (Fig. S5). Using these equations, it is possible to calculate  $J$ ,  $E$ ,  $R_0$  and  $r$  for every interaction. Data were reported in Table 3.

	<b>J (cm<sup>3</sup> L mol<sup>-1</sup>)</b>	<b>E (%)</b>	<b>R<sub>0</sub> (nm)</b>	<b>r (nm)</b>
<b>A</b>	$6.87 \cdot 10^{10}$	0.85	0.72	0.54
<b>B</b>	$8.06 \cdot 10^{11}$	0.95	1.09	0.65
<b>C</b>	$3.13 \cdot 10^{12}$	0.96	1.37	0.89
<b>D</b>	$6.16 \cdot 10^{12}$	0.73	1.52	1.29

Table 3 Parameters of  $J$ ,  $E$ ,  $R_0$  and  $r$  of HSA-drugs complexes at 296 K.

The distance  $r < 7$  nm indicates that the energy transfer between protein and drugs occurred with a high possibility [16, 23]. This is in agreement with conditions of Förster's non-radiative energy transfer theory [31], indicating again the static quenching interaction between protein and drugs. According to Stern-Volmer plots, data obtained with different methods are comparable each other.

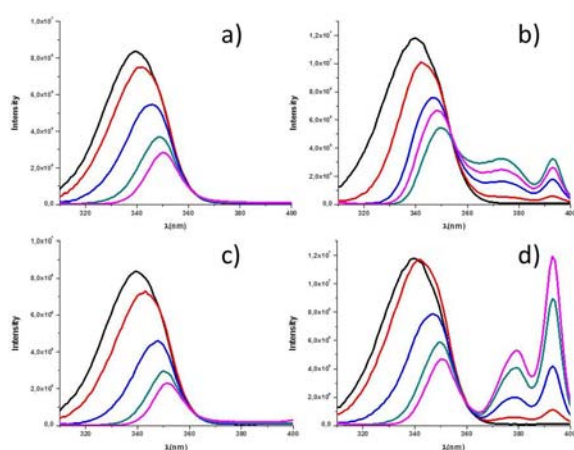
### 3.7 Conformation investigation

Synchronous fluorescence spectroscopy introduced by Lloyd has been used to investigate the conformational change of proteins [32]. The synchronous fluorescence spectrum could be obtained

by synchronously scanning the excitation and emission monochromators with a wavelength difference between excitation and emission as a constant. The intrinsic fluorescence of HSA is manifested by emission of Trp and Tyr residues present in the protein [33].

The synchronous fluorescence spectra obtained with  $\Delta\lambda = 60$  nm exclusively characterize the fluorescence of tryptophan residue. After complex formation, the local environment could change and induce a red or blue shift of the tryptophan emission spectra. The shift in the position of fluorescence emission maximum corresponds to changes of the polarity around the chromophore molecule. A blue shift of  $\lambda_{\max}$  means that the amino acid residues are located in a more hydrophobic environment and are less exposed to the solvent, while a red shift of  $\lambda_{\max}$  implies that the amino acid residues are in a polar environment and are more exposed to the solvent [34]. By synchronous fluorescence spectral changes of amino acid residues, the conformational changes of protein can be predicted. As well, we can obtain the information about the location of corticosteroids binding site from the synchronous fluorescence data.

The synchronous fluorescence spectra of HSA in presence of betamethasone, flunisolide, prednisolone and triamcinolone were recorded and shown in Fig 6.



**Fig.6** Synchronous fluorescence spectra of HSA-drugs at 296 K;  $\Delta\lambda = 60$  nm; [HSA]: black line; [Drugs]: (50.0- 800.0)  $\mu\text{M}$ .

For every analysed complex, the emission maximum of tryptophan residues showed significant

red shift of tryptophan residue fluorescence, confirming that every analysed drugs reached subdomain IIA, where only one Trp residue (Trp 214) in HSA is located. High concentration of drugs makes protein molecules extend, thus reducing energy transfer between aminoacids, reducing the fluorescence intensity [35]. Since Trp 214 is at site I, these results indicated that corticosteroids can bind to HSA in the hydrophobic cavity (site I) on subdomain IIA, which are in full agreement with quenching and competitive binding experiments.

#### 4. CONCLUSIONS

Human serum albumin is the main binding protein of plasma. Drug binding to HSA is a major problem in pharmaceutical research because the binding to albumin influences the effective drug concentration that can reach the target site. In this work, the interaction of albumin with four different corticosteroids was investigated at different temperatures by different spectroscopic approaches. UV–Vis spectroscopy confirmed that all the investigated drugs can bind to HSA to form a protein–drug complex. Quenching fluorescence data revealed that the protein can be bound by the studied corticosteroids, probably around the Trp 214 (as confirmed by competitive binding experiments and synchronous fluorescence) and that the quenching is governed by a static quenching (data are comparable with results obtained by FRET). According to thermodynamic parameters (negative  $\Delta H$  and  $\Delta S$  values) the hydrogen bonds and van der Waals forces play a major role in the binding process between albumin and betamethasone, flunisolide and prednisolone, while hydrophobic forces may play a major role in stabilizing albumin-triamcinolone complex. The evaluation of the equilibrium association ( $K_A$ ) and dissociation ( $K_D$ ) constants was obtained by a non-linear method at different temperatures. The data showed that temperature does not influence the formation of HSA-triamcinolone complex, while it can influence the interaction between albumin and betamethasone, flunisolide and prednisolone. This means that the drug structure may play a crucial role in the binding

with the protein. Usually, drugs bind to HSA high-affinity sites with typical association constants in the range of  $10^4$ – $10^6$   $M^{-1}$  [36]; as reported in literature [8], serum analysis revealed that the present corticosteroids can bind the HSA in a range between 70% and 80% thus with a low affinity. These data are in agreement with the equilibrium association constants obtained in this study. Usually a binder with high affinity, such as warfarin, shows  $K_A$  around  $10^5$   $M^{-1}$  [37] while the studied ICS have  $K_A$  in the order of  $10^3$   $M^{-1}$ .

The present spectroscopic approach offers a fast screening method to eventually investigate the structure-activity relationship (SAR) of new therapeutic molecules since it can discriminate the binding affinity with simple and reliable experiments.

## 5. ACKNOWLEDGEMENTS

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