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ORIGINAL ARTICLE

Interactions of acidic pharmaceuticals with human serum albumin: insights into the molecular toxicity of emerging pollutants

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Abstract Acidic pharmaceuticals such as diclofenac (DCF), clofibric acid (CA) and ketoprofen (KTP) have been detected frequently in environmental media. In order to reveal the toxicity of such emerging pollutants, their interactions with human serum albumin (HSA) were investigated by capillary electrophoresis, molecular spectrometry, and equilibrium dialysis. The binding constants and sites of these acidic pharmaceuticals with HSA were obtained. The thermodynamic parameters, e.g. enthalpy change and entropy change of these interactions were calculated to characterize that all the reactions resulted from hydrophobic and electrostatic interactions. The static quenching of the fluorescence of HSA was observed when interacted with acidic pharmaceuticals, indicating acidic pharmaceuticals bound to Tryptophan residue of HSA. The 3D fluorescence and circular dichroism confirmed that the secondary conformation of HSA changed after the interactions with the pharmaceuticals. At physiological condition, only 0.12 mM acidic pharmaceuticals reduced the binding of vitamin B_2 to HSA by 37, 30 and 21% for DCF, KTP and CA, respectively. This work provides an insight into non-covalent interactions between emerging contaminants and biomolecule, and is helpful for clarifying the toxic mechanism of such emerging contaminants.

Keywords HSA · Non-covalent interaction · Acidic pharmaceuticals · Fluorescence · Capillary electrophoresis

Introduction

In recent years, pharmaceuticals and personal care products (PPCPs) have caused a wide concern due to their continuous release to environment and the potential toxic effect on living organism, ecosystem, and public health (Daughton and Ternes 1999; Santos et al. 2010). Diclofenac (DCF), ketoprofen (KTP), and clofibric acid (CA) classified as acidic pharmaceuticals are frequently detected in the effluent of sewage treatment plants and surface, ground and drinking waters (Heberer 2002; Kolpin et al. 2002). DCF induced cytological changes of brown trout, damaged the renal function of rainbow trout, inhibited the reproduction algal and marine phytoplankton (DeLorenzo and Fleming 2008; Cleuvers 2003; Hoeger et al. 2005; Schmitt-Jansen et al. 2007). KTP affected membrane integrity in *Nitrosomonas europaea* (Wang and Gunsch 2010). CA was frequently used to assess toxicity due to its high persistence existing in environment. It had an acute toxic effect on bacteria, ciliates, daphnids and fish embryos (Ferrari et al. 2003), exhibited an alteration in the reproductive function of fathead minnow fish (Triebskorn et al. 2007) and induced the cytological changes in gills of rainbow trout (Runnalls et al. 2007). In fact, all the toxic effects on the cell, organ, and tissue were originated from the molecular-level reaction and change. Therefore, it was imperative to investigate the interaction between biomolecule and exogenous chemicals in vitro, which was significant to reveal the toxic effect mechanism of the exogenous chemicals.

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Human serum albumin (HSA) is the most abundant protein in the circulatory system with a physiological concentration of 35–45 g/L, and plays a key role in the transport of a variety of endogenous and exogenous ligands, e.g. acidic pharmaceuticals (Labieniec and Gabryelak 2006). The distribution, transportation, and metabolism of the ligands in the blood are significantly affected by their binding to HSA because they may cause the conformational change of protein and then the potential effect on its normal physiological function. Therefore, the interactions between HSA and acidic pharmaceuticals are of imperative and fundamental importance. Some methods were developed to determine these interactions, e.g. equilibrium dialysis (Wu et al. 2010), ITC (Chen et al. 2009), ultrafiltration (Heinze and Holzgrabe 2006), HPLC (Singh and Mehta 2006), gel filtration (Alebic-Kolbah et al. 1979), microdialysis (Wang et al. 1998), circular dichroism (CD) (Zhang et al. 2009a), fluorescence spectrometry (Ge et al. 2011) and capillary electrophoresis (CE) (Tanaka and Terabe 2002). Besides, molecular spectrometry such as fluorescence is the conventional methods applied to investigate the interaction between HSA and ligands due to the rapidity, sensitivity and ease of implementation. From change of HSA spectra before and after the addition of ligands, a great number of information can reflect the binding mechanism and mode. The interactions of acidic pharmaceuticals with HSA were investigated by capillary electrophoresis-frontal analysis (CE-FA) and molecular spectrometry. CE-FA has the advantage of low sample consumption, relatively short time and ease of automation (Østergaard et al. 2002). Furthermore, the secondary structure change of HSA was studied by circular dichroism, and the effect of acidic pharmaceuticals on the transport function of HSA was studied by equilibrium dialysis.

Materials and methods

Instruments and chemicals

The capillary electrophoresis experiments were performed on the P/ACE MDQ capillary electrophoresis system (Beckman, Coulter, Fullerton, CA, USA) with a UV detector. The uncoated fused-silica capillary of 75 μm i.d. was purchased from Polymicro Technologies Inc. (Phoenix, AZ, USA), and capillary length to detector was 50 cm. The fluorescence spectra measurement was carried out on a F-4500 fluorophotometer (Hitachi, Japan) equipped with 1 cm quartz cell and a thermostat bath. The circular dichroism experiment was performed on a J-715 CD spectropolarimeter (Jasco Instruments, Tokyo, Japan). Model RC 30-5K semi-permeable membranes (Molecular Weight Cut Off 5 kDa, Shanghai Green Bird, China) were used in equilibrium dialysis experiment.

All reagents are of analytical grade. HSA (essentially fatty acid free), DCF, KTP, CA, and Vitamin B_2 were purchased from Sigma-Aldrich Company. Sodium hydroxide, hydrochloric acid, potassium dihydrogen phosphate and disodium hydrogen phosphate were obtained from Sinopharm Chemical Reagent Co., Ltd. A 67 mM phosphate buffer (pH 7.4, I 0.17) was prepared by dissolving an appropriate amount of potassium dihydrogen phosphate and disodium hydrogen phosphate in deionized water. A HSA stock solution was prepared by dissolving appropriate amount of protein powder in the phosphate buffer and stored at 4°C prior of use. The stock standard solutions of acidic pharmaceuticals were prepared in the phosphate buffer. All the working solutions were obtained by diluting the stock solutions with the phosphate buffer.

Capillary electrophoresis analysis of the HSA-acidic pharmaceuticals interactions

A new capillary was conditioned with 1 M NaOH at 60°C for 10 min, then flushed with deionized water and phosphate buffer for 10 min at 36.5°C, respectively. At the beginning of each day and during the runs, the capillary was conditioned at 0.5 psi as following sequence: 3 min rinse with deionized water, followed by 3 min rinse with 1 M NaOH and 3 min rinse with deionzed water, finally 3 min rinse with phosphate buffer. Such conditioning could basically eliminate the influence of the adsorbed protein in the capillary.

The running buffer in all CE experiments was 67 mM phosphate buffer. All the solutions injected in the capillary were filtered through 0.22-μM nylon membrane before use. Series of mixtures with different concentrations of acidic pharmaceuticals in the range between 20 and 950 μM and a constant HSA concentration of 25 μM were prepared by dilution of the stock solutions to the required concentrations with the running buffer. After incubated for 15 min, the mixtures were injected into the capillary with the pressure of 0.8 psi for 60 s at the positive side. The separation voltage was set at 18 kV, and the detected wavelength was set at 214, 254 and 280 nm for DCF, KTP and CA, respectively. Capillary temperatures were kept at 298, 303 and 309.5 K to investigate the thermodynamic parameters.

Fluorescence quenching of HSA

A 2.5 ml solution containing 4.00-μM HSA was added to the quartz cell, then titrated by successive addition of the stock solutions of pharmaceuticals with the trace syringes. The excitation and emission slits of the passage of band

were set at 10 and 20 nm, respectively, and the scanning speed was kept at 2,400 nm/min. Fluorescence quenching spectra of HSA were excited at 295 nm, and then recorded at emission wavelength of 315–450 nm. All fluorescence measurements were corrected against the inner filter effect using the equation (Ding et al. 2009): $F_{cor} = F_{obs} \times e^{\frac{A_{ex}+A_{em}}{2}}$, where F_{cor} and F_{obs} were the fluorescence intensities corrected and observed, respectively, whereas A_{ex} and A_{em} were the adsorbance at excitation and emission wavelength, respectively.

3D fluorescence measurement of HSA in the presence of acidic pharmaceuticals

The parameters of 3D fluorescence spectra measurement were set as follows: the excitation wavelength was set between 200 and 350 nm with the increment of 10 nm, and the emission wavelength was recorded in the range between 200 and 500 nm; the scan speed was set at 12,000 nm/min; both the excitation and emission slits with a band pass were set at 5 nm. The 3D fluorescence spectra of HSA (2.00 μM) were recorded in the absence and the presence of acidic pharmaceuticals (1:1).

CD of HSA in the presence of acidic pharmaceuticals

Circular dichroism was performed on a spectropolarimeter with a 0.1-cm path length quartz cell. The CD spectra were recorded in the range of 190–260 nm with 0.1-nm data pitch, and averaged over three scans with the scanning speed of 100 nm/min. From the CD spectra, the relative contents of secondary structure forms of HSA, α-Helix, β-pleated sheet, β-turn and random coil, were calculated by the secondary structure estimation-standard analysis measurement software of the spectropolarimeter.

Effect of acidic pharmaceuticals on the physiological function of HSA

Equilibrium dialysis was conducted to reveal the effect of acidic pharmaceuticals on the physiological function of HSA to transport vitamin B_2 (VB_2) with a special dialysis device designed by Gao's group (Zhang et al. 2009a). 12.5 ml of solution containing phosphate buffer, 0.025 mM HSA, 0.076 mM VB_2, and 0–0.12 mM acidic pharmaceuticals were added into the dialysis bag, which was merged in 37.5 ml of dialysate solution containing phosphate buffer. After 10 h at 36.5°C, 2.5 ml of dialysate solution was sampled, and the VB_2 concentration determined with a fluorospectrophotometer. The excitation and emission wavelength were at 440 and 525 nm, respectively. The fluorescence intensity of VB_2 in the dialysis solution was recorded to quantify the content of VB_2 and then calculate the binding ratio of VB_2 to HSA.

Results and discussion

Application of capillary electrophoresis to the HSA—acidic pharmaceuticals interactions

Time required to reach equilibrium before injection

Before the sample injected in the capillary, the binding reaction between HSA and ligands should achieve equilibrium. Hence, time required to reach equilibrium was investigated before injection. For this purpose, the samples incubated for different periods of time (5, 15, 45, 80 and 150 min) were successively injected into the capillary, the calculated free concentrations of DCF were found to be almost unchanged (Fig. S1 of the Electronic supplementary material), indicating the binding equilibrium achieved quickly after the addition of DCF to HSA solution. For drug-plasma protein system, binding equilibrium was achieved almost instantaneously due to the high association and dissociation rates of the drug-protein complex (Vuignier et al. 2010). Therefore, specific incubation time was not necessary before injection. In order to keep the sample temperature same as the capillary, all the samples were incubated for 15 min at the corresponding temperature in the further experiments.

Effect of injection time

Although the formation of plateau may not be absolutely required to estimate binding parameters of ligands and HSA correctly, the presence of plateau peaks may lead to a more robust method of analysis. Because the plateau height is not so affected by changes in migration times, EOF, length of the capillary, and applied voltage as in the case of a zonal peak (Østergaard and Heegaard 2003). Hence, it is necessary to obtain a plateau peak in the CE-FA by the introduction of a relatively large volume of sample. In order to investigate the effect of different injection times on forming plateau, the same solution containing 25-μM HSA and 150-μM DCF was injected in the capillary electrophoresis at different injection times of 5, 10, 20, 30, 40, 60 s, and the corresponding electropherograms were demonstrated in Fig. 1. A plateau of free DCF began to appear at an injection time of 30 s, while the plateau was not flat enough. A more evident and flat plateau was obtained at the injection time of 60 s. Furthermore, with the increase of the injection time, the plateau became broader, while the height of the plateau was almost

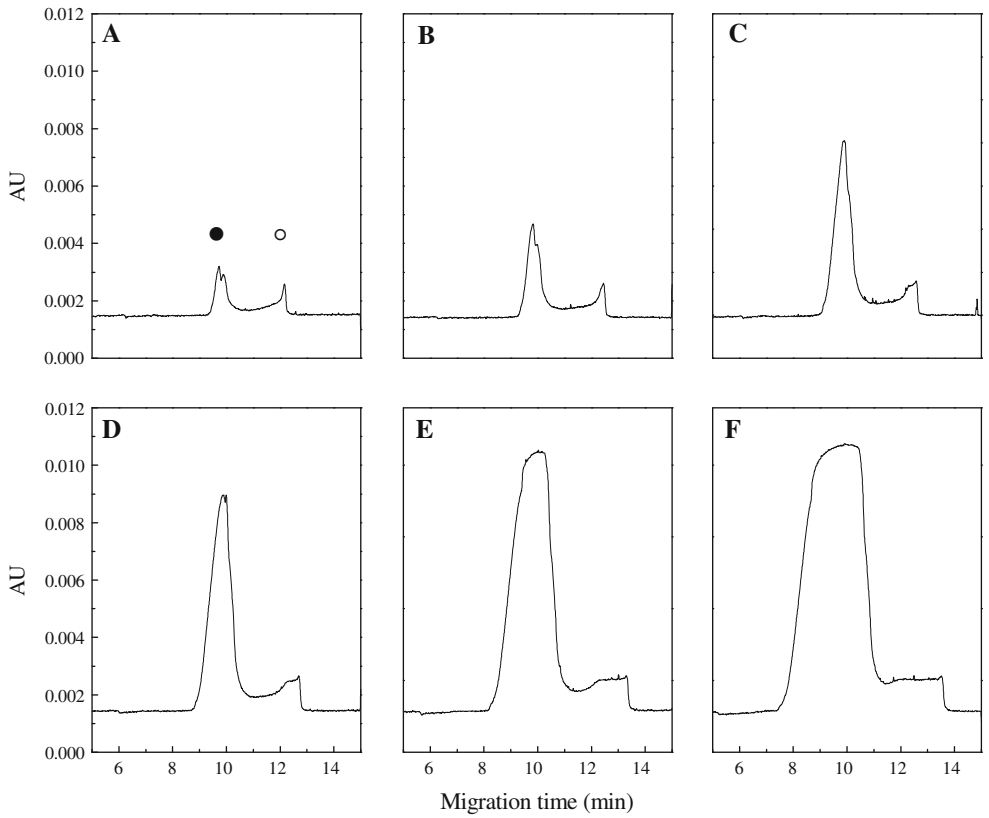

Fig. 1 CE gram of the solutions containing HSA (25.06 μM) and DCF (121.6-μM DCF) at pH 7.4 and 36.5°C. Injection by pressure of 0.5 psi for **a** 5 s; **b** 10 s; **c** 20 s; **d** 30 s; **e** 40 s and **f** 60 s. *Filled circle* HSA and HSA-DCF complex; *open circle* DCF

constant. Thus, an injection time of 60 s was chosen for the further capillary electrophoresis experiments.

Linearity and precision of acidic pharmaceuticals

Series of concentrations of acidic pharmaceuticals were injected into the capillary. The concentrations of the pharmaceuticals were linearly regressed with the corresponding plateau peak height (DCF: $y = 41.568x + 431.56$; KTP: $y = 74.225x - 89.753$; CA: $y = 45.96x - 373.92$). The calibration curves had good linearity due to the correlation coefficient (R^2) greater than 0.997. The relative standard deviation (RSD) was calculated to be smaller than 1.8%.

Binding of acidic pharmaceuticals to HSA

The interactions of three acidic pharmaceuticals with HSA were conducted under the optimized conditions. The mixtures of different concentrations of acidic pharmaceutical and a constant concentration of HSA were injected in the capillary, and the electrophoregram of the interaction was illustrated in Fig. 2 with DCF and HSA as an example. During separation process, free DCF, HSA, and DCF-HSA complex migrated towards the detector together due to the electro osmotic flow. Because the electrophoretic mobility of HSA was not varied after binding of low-molecular ligands (Hage and Austin 2000), the mobility of DCF-HSA complex could be supposed to be equal to that of HSA, namely, the bound DCF moved with the same mobility as HSA. Because of the larger negative electrophoretic mobility, the free DCF fell behind the HSA zone, and the plateau corresponding to free DCF was formed (Fig. 2a), the height of which was proportional to the concentration of free DCF in the original sample. After injection the solution containing only DCF (Fig. 2b), the plateau appeared as well, and the height of plateau was decreased with the addition of HSA. The decreased part was corresponding to the bound DCF moved along with the HSA zone, which could be determined according to the calibration line. The binding curves of DCF, CA and KTP were plotted as the number of bound pharmaceutical molecules per HSA molecule (r) versus the free concentration (C_f), as demonstrated in Fig. 3. The binding constants (K) and binding sites (n) could be obtained by analyzing the experimental data with the non-linear regression according to the following equation (Tanaka and Terabe 2002):

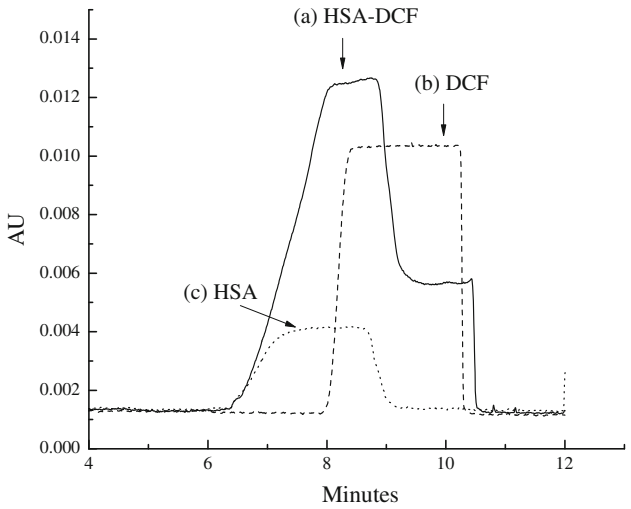

Fig. 2 Comparison of the electrophoregrams of DCF with HSA. Samples containing 25.06-μM HSA and 222.9-μM DCF (**a**), 222.9-μM DCF (**b**), and 25.06-μM HSA (**c**)

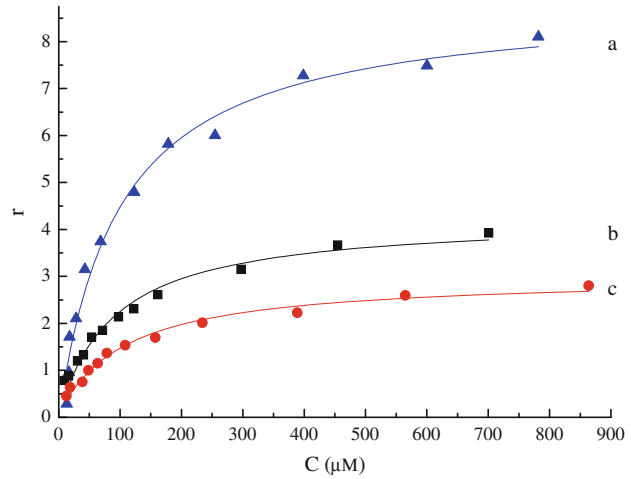

Fig. 3 Effects of acidic pharmaceuticals binding to HSA. **a** DCF, **b** KTP, **c** CA

$$r = \frac{C_b}{P} = \frac{C_t - C_f}{P} = \frac{nKC_f}{1 + KC_f} \quad (1)$$

where P is the concentration of HSA, C_b is the molarity of bound acidic pharmaceuticals, C_t is the molarity of total pharmaceuticals, C_f is the molarity of free pharmaceuticals, r is the molar ratio of the acidic pharmaceuticals binding to HSA. For the DCF, KTP and CA reactions with HSA, K was calculated to be 3.36×10^4, 1.12×10^4, and 9.47×10^3 M^{-1} and n to be 8.1, 4.2, and 3.0. It is well known, various non-covalent interactions, e.g. hydrogen bond, van der Waals force, electrostatic and hydrophobic ones may exist simultaneously between ligand and protein, in which the electrostatic attraction often plays an inductive role, i.e. pulling the ligand charged to approach HSA (Wu et al. 2009). And also, such a joint interaction changes the secondary conformation of protein (Zhang et al. 2009a). It provides a clear understanding why the inhibition of protein activity occurs in a drug medium (Xu et al. 2010). From the dissociation constants (K_a) of some amino acid residues of HSA (K_a = 10.53 for Lys, 12.48 for Arg), the side groups of some polar amino acid residues will be protonated and positive in neutral solution. Concerning the presence of high polar carboxyl group in the studied chemicals, the electrostatic attraction between the dissociated negatively charged carboxyl group and positively charged amino acid residues of HSA will play an important role in the binding. The molecular modeling and computational mapping also confirmed several polar amino acid residues could stabilize the negatively charged molecules (Zsila et al. 2003). Moreover, the hydrophobic interaction will also occur between the benzene ring of the studied chemicals and the non-polar side groups of amino acid residues of HSA. Because of the common characteristics of the three acidic pharmaceuticals, e.g. the presence of benzene ring and carboxyl group in the chemical structures, their binding constants to HSA were almost in the same order of magnitude. While the different part in the chemical structure might account for the difference of binding constants and sites. There are two benzenes in DCF molecule with two chlorine atoms on the side, thus its hydrophobicity is larger than KTP and CA. Hence, the hydrophobic interaction between DCF and non-polar side groups of amino acid residues is expected to be stronger, and the binding constants to be larger.

Thermodynamic parameters are important for understanding the mechanism of biomolecule-ligand reaction. In order to evaluate the binding force between acidic pharmaceuticals and HSA, the thermodynamic parameters, including standard enthalpy change (ΔH^0), entropy change (ΔS^0) and free energy change (ΔG^0) of the interaction were evaluated. The thermodynamic parameters could be calculated with the following equations:

$$\ln K = -\Delta H^0/RT + \Delta S^0/R \quad (2)$$

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 = -RT \ln K \quad (3)$$

where K is binding constant at the corresponding temperature, R is the gas constant. According to the above equations, the thermodynamic parameters were calculated and presented in Table 1. The negative value of free energy change (ΔG^0) indicated the spontaneous interaction between HSA and acidic pharmaceuticals. All of the ΔH^0 values were much less than 60 kcal/mol, so the acidic pharmaceuticals-HSA interactions were non-covalent (Wu et al. 2009). Furthermore, Ross and Subramanian have characterized the sign and magnitude of the thermodynamic parameters related to the various kinds of

Table 1 Thermodynamic parameters for the interaction between acidic pharmaceuticals and HSA

Chemicals	T (K)	K (M^{-1})	ΔH (kJ/mol)	ΔS (J/mol K)	ΔG (kJ/mol)	R^2
DCF	298	37,114.2	−6.6	+65.5	−26.1 to −26.8	0.9921
	303	35,209				
	309.5	33,619				
KTP	298	13,086.4	−10.5	+43.6	−23.5 to −24.0	0.9896
	303	12,358				
	309.5	11,190				
CA	298	11,251	−11.4	+39.2	−23.1 to −23.6	0.9892
	303	10,261				
	309.5	9,470				

interactions in the protein association process (Ross and Subramanian 1981). The positive entropy change (ΔS^0) was often taken as the evidence for the hydrophobic interactions, which might be attributed to the presence of benzene ring in the chemicals. The negative ΔH^0 and positive ΔS^0 values were characteristic of specific electrostatic interactions. Concerning ΔH^0 was less than $-T\Delta S^0$, all the reactions were expected to be driven by entropy change. Consequently, it was impossible to elucidate the thermodynamic parameters of acidic pharmaceuticals-HSA binding reaction on the aspect of a single intermolecular binding mode. It was more likely that hydrophobic interactions dominated in the interaction, while electrostatic interaction also involved.

Human serum albumin is a globular protein which consists of 585 amino acids. The crystallographic analysis of HSA reveals that the protein is a heart-shaped molecule, containing three structurally similar domains (I, II, III), each of which is further divided into two subdomains (A and B) (Trnkova et al. 2010). All the helixic subdomains are distributed round a hydrophobic intracavity and the hydrophilic side groups of HSA exposed on the outer surface. During protein–ligand interaction, polar bonds are likely to be formed outside of the hydrophobic cavities due to the polar residues predominated here, and the reaction is always exothermic; while hydrophobic interaction might occur inside the hydrophobic cavity because of the non-polar side groups of amino acid residues there, and the reaction is endothermic with decreasing of entropy change. Because there are polar carboxyl group and hydrophobic benzene ring existing in the studied chemicals, the acidic pharmaceutical molecules were supposed to bind to HSA on the surface and inside the cavity. They bound to the surface of HSA via electrostatic attraction between their carboxyl group and polar amino acid residues of HSA. After the binding sites of HSA surface were occupied, the acidic pharmaceutical molecules entered the hydrophobic cavity and the hydrophobic benzene ring reacted with the non-polar amino acid residues. According to the structures of the three studied chemicals, DCF was more hydrophobic than KTP and CA, which could also be speculated from their octanol–water partitioning coefficients (log*Kow*: DCF 4.5, KTP 3.12, CA 2.57). Therefore, more DCF molecule would enter the hydrophobic cavity and then bound to the non-polar amino acid residues, thus the entropy change ΔS was supposed to be larger compared to KTP and CA. On the contrary, the dissociation coefficient of CA was smaller than DCF and KTP (pK_a: CA 3.0, KTP 4.5, DCF 4.15), indicating CA was more negative in the neutral condition, so the electrostatic interaction between CA and polar amino acid residues of HSA was stronger, and the enthalpy change (ΔH) was expected to be larger than DCF and KTP.

Spectroscopic characterization of the HSA-acidic pharmaceuticals interactions

Fluorescence quenching is a decrease of the quantum yield of fluorescence from a fluorophore induced by various molecular interactions, such as exciting-state reactions, molecular rearrangements, energy transfer, collisional quenching and ground-state complex formation (Zhang et al. 2009b). In general, the different quenching mechanisms are classified in two types of quenching: static quenching and dynamic quenching. Static quenching is resulted from the formation of ground-state complex, while the dynamic quenching is due to collisional encounters between the fluorophore and quencher.

In order to investigate the interaction between the acidic pharmaceuticals and HSA, the fluorescence emission spectra of HSA in the range between 315 and 450 were recorded with the addition of DCF, KTP, and CA at the excitation wavelength of 295 nm, the corresponding spectra were presented in Fig. 4. HSA had a strong fluorescence emission peak at 341 nm. With the addition of acidic pharmaceuticals, the fluorescence intensity of HSA gradually decreased and the peak shapes did not change, indicating that the interaction between the acidic

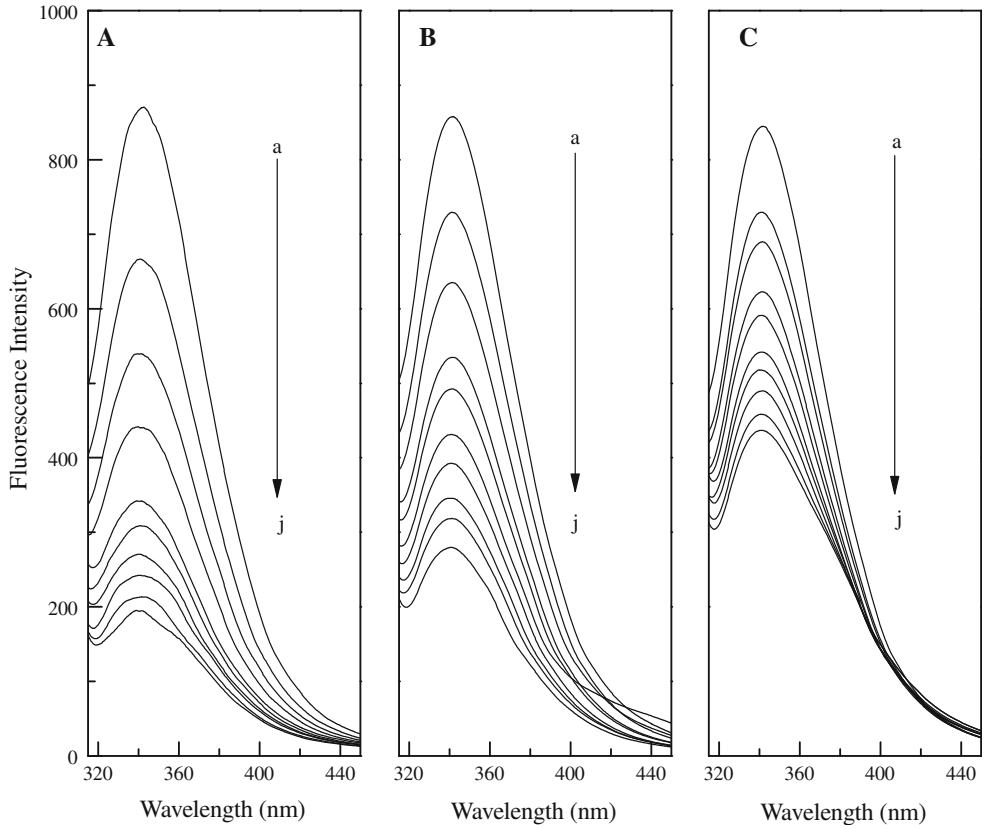

Fig. 4 Effect of acidic pharmaceuticals on the fluorescence emission spectra of HSA. **a** 4-μM HSA and DCF ($a \rightarrow j$): 0, 2, 4, 6, 8, 10, 12, 14, 16, 18 μM, **b** 4-μM HSA and KTP ($a \rightarrow j$): 0, 3, 6, 9, 12, 15, 18, 21, 24, 27 μM, **c** 4-μM HSA and CA ($a \rightarrow j$): 0, 12, 24, 36, 48, 60, 72, 84, 96, 108 μM

pharmaceuticals and HSA resulted in the quenching of the intrinsic fluorescence of HSA. In order to confirm the quenching mechanism, the fluorescence data were analyzed by the well-known Stern–Volmer equation:

$$\frac{F_0}{F} = 1 + K_{SV}[Q] = 1 + k_q\tau_0[Q], \quad (4)$$

where F_0 and F are the fluorescence intensities of HSA before and after the addition of quencher, respectively. $[Q]$ is the concentration of the quencher. K_{SV} is the Stern–Volmer dynamic quenching constant, τ_0 is excited state life time of the fluorophore in the absence of quencher and the τ_0 of the biopolymer is 10^{-8} s. k_q is the bimolecular quenching rate constant, for dynamic quenching, the maximum scattering collision quenching constant of various quenchers is 2×10^{10} L mol^{-1} s^{-1} (Eftink and Ghiron 1981). The values of k_q were calculated to be 2.1×10^{14}, 5.7×10^{13}, 1.3×10^{13} L mol^{-1} s^{-1} for DCF, KTP and CA, which were much larger than 2×10^{10} L mol^{-1} s^{-1}, indicating the fluorescence quenching of HSA was initiated by the static quenching instead of dynamic quenching.

The fluorescence data were further analyzed using the modified Stern–Volmer equation:

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a} + \frac{1}{K_a f_a[Q]}, \quad (5)$$

where f_a is the fraction of accessible fluorescence, K_a is the effective quenching constant of the accessible fluorophore. A linear regression of $F_0/(F_0 - F)$ versus $1/[Q]$ was conducted, with the slope and intercept corresponding to $(K_a f_a)^{-1}$ and $(f_a)^{-1}$. The K_a values of HSA quenched by acidic pharmaceuticals were calculated to be 9.25×10^5, 1.36×10^5, and 3.2×10^4 M^{-1} for DCF, KTP and CA, respectively. HSA has one tryptophan residue which significantly contributes to the intrinsic fluorescence of the protein, located in the hydrophobic pocket of subdomain IIA. After the acidic pharmaceuticals insert into the hydrophobic pocket of subdomain IIA, the hydrophobic part of the molecules interacted with tryptophan residue, and then quenched its intrinsic fluorescence. Because the hydrophobicity of DCF was larger than KTP and CA. The hydrophobic interaction between DCF and tryptophan residue was expected to be stronger, and the effective quenching constants to be larger, which accounted for the decrease trend about the values of K_a for HSA quenched by DCF, KTP, and CA.

Change of HSA conformation

Three-dimensional fluorescence measurement has been used to investigate the conformational change of protein interacting with small molecules in recent years (Mote et al. 2010). The information about fluorescence characteristics can be obtained by changing the excitation and emission wavelength simultaneously. It can be suggested conformational changes of protein that the excitation or emission wavelength around the fluorescence peak shift towards higher or lower wavelength regions, or a peak appears or disappears in the spectra. In order to obtain the information about the conformational change of HSA in the presence of acidic pharmaceuticals, the 3D fluorescence spectra were recorded before and after the addition of acidic pharmaceuticals, and the spectra were displayed in Fig. 5 with the corresponding parameters presented in Table 2. From Fig. 5, peaks *a* and *b* represented Rayleigh scattering peak and the second-ordered scattering peak. Peak 1 mainly revealed the fluorescence characteristics of Trp and Tyr residues, because the excitation of HSA at 280 nm presented the intrinsic fluorescence information of Trp and Tyr, while the fluorescence of Phe residue was too small to be neglected (Ding et al. 2009). Peak 2 exhibited the fluorescence behavior of polypeptide chain backbone structures, because the excitation of HSA at 230 nm brought about the transition of $n \rightarrow \pi^*$ of the polypeptide backbone structure C=O (Glazer and Smith 1961). After

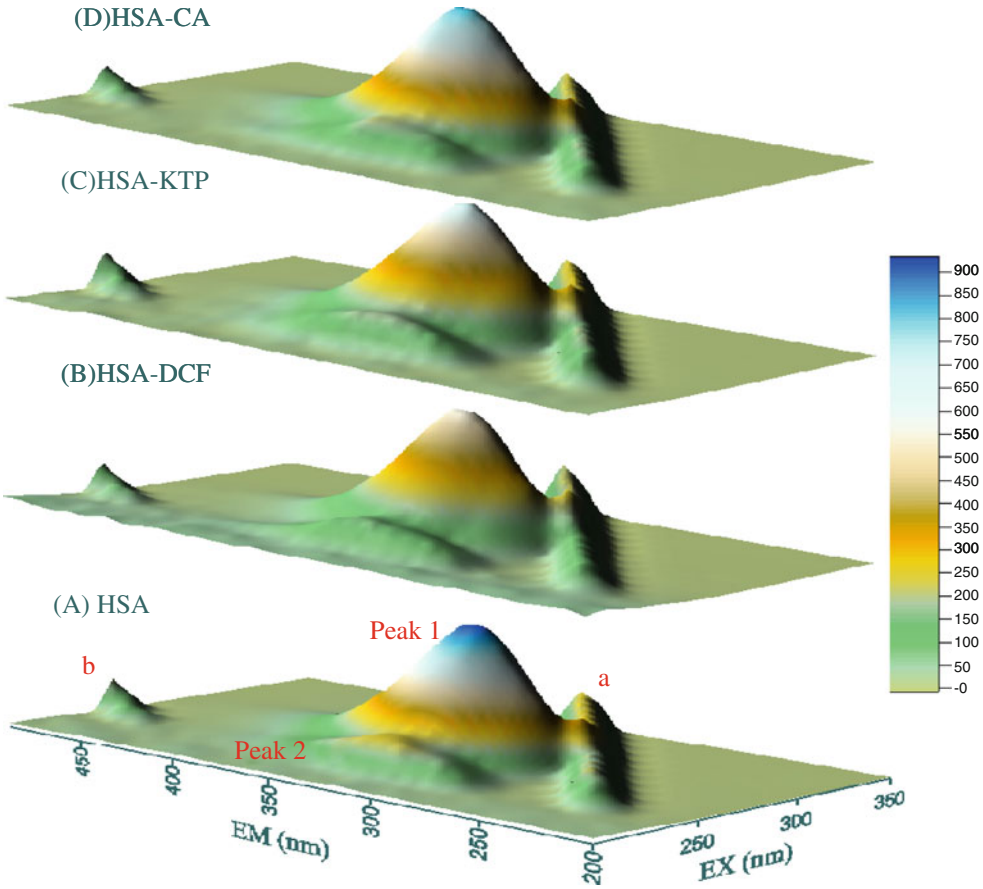

Fig. 5 Three-dimensional fluorescence spectra of HSA and HSA-acidic pharmaceuticals system

Table 2 Three-dimensional fluorescence spectral characteristics of HSA and HSA-acidic pharmaceuticals system

Reaction system	Peak 1			Peak 2		
	Peak position $\lambda_{em}/\lambda_{ex}$ (nm/nm)	Stokes ($\triangle\lambda$)	Intensity	Peak position $\lambda_{em}/\lambda_{ex}$ (nm/nm)	Stokes ($\triangle\lambda$)	Intensity
HSA	280/332	52	900.6	230/340	110	213.7
HSA-DCF	280/335	55	520.4	230/341	111	146.2
HSA-KTP	280/340	60	618.1	230/342	112	148.1
HSA-CA	280/338	58	764.1	230/341	111	173.3

the addition of DCF, KTP and CA, the fluorescence intensity of peak 1 and peak 2 decreased obviously but to various degrees: peak 1 decreased by 42, 31, and 15%, while peak 2 decreased by 31, 30, and 19% for DCF, KTP, CA, respectively. Moreover, the maximum emission wavelength of peak 1 and peak 2 shifted. All these phenomena indicated that acidic pharmaceuticals bound to HSA resulted in the fluorescence quenching of HSA, the change of hydrophobicity in the vicinity of amino acid residue and the unfolding of polypeptide of HSA, which confirmed the alteration of the conformation of HSA.

To further understand the alteration of the secondary structure of HSA after the addition of acidic pharmaceuticals, CD was measured to monitor the change of HSA conformation. The CD spectrum of HSA exhibited two negative bands in the far-UV region at 208 and 222 nm (Fig. S2 of the Electronic supplementary material), which was the characteristic of α-helix in the advanced structure of protein. After the addition of acidic pharmaceuticals, the CD spectrum of HSA changed, and the corresponding contents were given in Table 3. In the presence of acidic pharmaceuticals, the α-helix fraction increased, whereas *β*-pleated sheet and random coil decreased, and the random coil even disappeared after the addition of CA. Obviously, the decrease of *β*-pleated sheet and random coil resulted in the increase of α-helix. Thus, the addition of acidic pharmaceuticals transformed some *β*-pleated sheet and random coil into α-helix.

Therefore, the interaction of acidic pharmaceuticals with HSA induced the unfolding of the polypeptides of protein, resulting in increasing the exposure of some hydrophobic regions that had been buried. Furthermore, acidic pharmaceuticals bound to the amino acid residues of the polypeptide chain and might destroy their hydrogen bonding networks, making HSA adopt a more loose conformation state (Cui et al. 2004). The secondary structure of protein was closely related to the biological activity of protein, thus the change of the secondary structure might influence the physiological function of HSA.

Table 3 The content of four secondary structures of HSA before and after the addition of acidic pharmaceuticals (0.2-μM HSA, 6-μM DCF, 6-μM KTP and 6-μM CA)

	HSA (%)	HSA-DCF (%)	HSA-KTP (%)	HSA-CA (%)
α-Helix	26.50	64.40	47.20	86.40
β-pleated sheet	46.40	29.10	36	13.60
β-turn	0.00	0.00	0.00	0.00
Random coil	27.00	6.60	16.80	0

Effect of acidic pharmaceuticals on the HSA physiological function

Vitamin B_2 is a water-soluble vitamin present in a variety of foods. It participates in a diversity of redox reactions central to human metabolism, through the cofactors FMN and FAD, which act as electron carriers. Inadequate intake of vitamin B_2 would be expected to lead to disturbance in steps in intermediary metabolism, with functional implications (Powers 2003). Serum albumin is the most abundant protein in blood plasma, which contributes to colloid osmotic blood pressure and chiefly responsible for the maintenance of blood pH. Furthermore, it is the major carrier protein in blood which can transport a variety of endogenous and exogenous ligands, e.g. drugs, vitamins, exogenous pollutants and so on. Acidic pharmaceuticals were bound to HSA by the joint non-covalent interactions and then changed the secondary conformation of HSA. Thus, the normal physiological function of HSA might affect, such as the transportation function. From change of the VB_2-transporting, the binding ratio of VB_2 to HSA decreased gradually with increase of acidic pharmaceuticals (Fig. 6). Only 0.12 mM acidic pharmaceuticals have reduced the binding number by 37, 30 and 21% for DCF, KTP and CA, respectively. One reason was that the pharmaceuticals occupied competitively the binding sites of VB_2, and the subsequent conformation change of HSA was unfavorable for binding of VB_2 to HSA. Moreover, the decrease tread about binding ratio of VB_2 to HSA for three acidic pharmaceuticals was similar to that of their different binding constants and sites. The binding constant of DCF to HSA was larger than that of KTP and CA, hence DCF comparatively occupied more binding sites of VB_2, resulting in the more decrease of binding ratio of VB_2 to

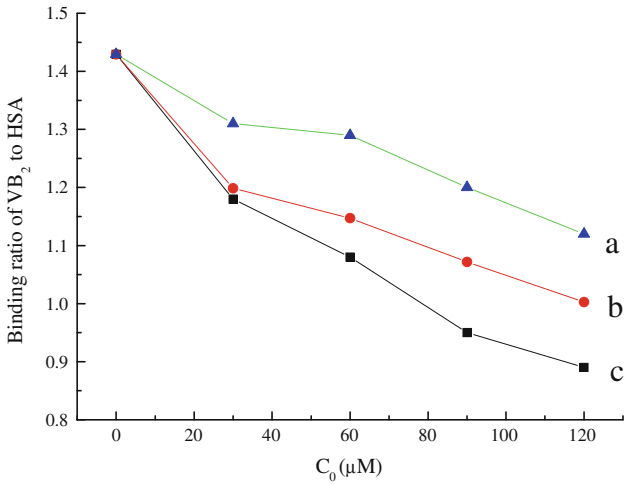

Fig. 6 Effects of acidic pharmaceuticals on the physiological function of HSA to transport VB_2. **a** DCF, **b** KTP, **c** CA

HSA. Therefore, non-covalent binding of such chemicals potentially affects the physiological function of protein by changing its conformation and overlapping its active sites (Zhang et al. 2009a).

The acidic pharmaceuticals in the environment were in the level of nM (Heberer 2002; Kolpin et al. 2002), which were much lower than that studied in this work. However, the acidic pharmaceuticals could be concentrated in the organisms which were long-term exposed in pharmaceutical contaminated environment. Oaks et al. (2004) have reported that the residues of diclofenac in the environment in Pakistan were concentrated in the local vulture and responsible for their renal disease. Therefore, excessive ingestion of pharmaceuticals or long-term exposure in pharmaceutical contaminated environment would potentially influence the normal physiological function of human protein.

Conclusions

Interaction between HSA and acidic pharmaceuticals was investigated by capillary electrophoresis and molecular spectrometry. The binding constants and sites of these acidic pharmaceuticals with HSA were obtained in the CE-FA analysis. The thermodynamic parameters indicated hydrophobic and electrostatic interactions were the main contributor that induced acidic pharmaceuticals binding to HSA. After interaction with acidic pharmaceuticals, the conformation of HSA was changed, with the binding ratio of VB_2 to HSA being reduced by 37, 30 and 21% for DCF, KTP and CA, respectively, indicating the physiological function of HSA to transport Vitamin B_2 was inhibited. Hence, excessive ingestion of such acidic pharmaceuticals or long-term drinking pharmaceutical contaminated water would influence the normal physiological function of human protein and enzyme, which would cause human health risk.

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