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Short communication

Interactions of human serum albumin with meloxicam Characterization of binding site

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ABSTRACT

Human serum albumin (HSA) is the most prominent protein in plasma. The three-domain design of HSA provides a variety of binding sites for many ligands, including heme, bilirubin and drugs. Here, we report the effect of new generation, non-steroidal anti-inflammatory drug (NSAID) meloxicam on the albumin conformation and ligand binding. In the present work the interaction of meloxicam with HSA in aqueous solution at physiological pH has been investigated through circular dichroism and fluorescence spectroscopy. The strong quenching of the fluorescence clearly indicated that the binding of the drug to HSA changed the microenvironment of tryptophan residue and the tertiary structure of HSA. This was confirmed by the destabilization of the warfarin binding site. CD and fluorescence spectroscopic results showed marked reductions (about 40% decrease in the CD Cotton effect intensity, and ~15% decrease of the fluorescence intensity) in the affinity of albumin for bilirubin upon meloxicam binding. The strong inhibition of warfarin and ANS bound to protein after meloxicam modification compared with aspirin confirms that the binding site of both drugs is not the same.

1. Introduction

Reversible attachment to serum proteins significantly modulates the pharmacokinetics and pharmacodynamics of many drugs. One of the most important factors affecting the distribution and the free, active concentration of many drugs is binding affinity for human serum albumin (HSA). HSA, a single-chain protein, is composed of three homologous, predominantly helical domains (I–III); each of these is comprised of two subdomains (A and B). Its amino acid sequence contains a total of 17 disulphide bridges, one free thiol (Cys 34) and a single tryptophan (Trp 214). Albumin interacts reversibly with a wide variety of endogenous and exogenous compounds [1,2]. The principal regions of ligand binding sites of albumin are located in hydrophobic cavities in subdomains IIA and IIIA, which exhibit similar chemistry. According to the Sudlow's nomenclature, bulky heterocyclic anions bind to site I (located in subdomain IIA), whereas site II (located in subdomain IIIA) is preferred by aromatic carboxylates with an extended conformation. Remarkably, ibuprofen, a non-steroidal anti-inflammatory agent, and warfarin, an anti-coagulant drug, are considered as stereotypical ligands for Sudlow's site II and Sudlow's site I, respectively [3,4]. Usually, drugs bind to high-affinity sites with typical association constants in the range of 10^4–10^6 M^{-1} Warfarin binds to Sudlow's site I in a pocket formed by the packing of all six helices of subdomain IIA [5,6]. The binding site has two subchambers that accommodate different portions of the warfarin molecule. The interaction between warfarin and albumin appears to be dominated by hydrophobic contacts, although specific electrostatic interaction is observed [5]. Warfarin shares this binding site with a range of other drugs (including phenylbutazone, tolbutamide and indomethacin) and thus competes with them for binding to HSA. The binding of drugs may change considerably the ability of the protein to bind other molecules, including endogenous ligand such as heme or bilirubin.

Bilirubin has been reported to bind to a dynamic and flexible site in subdomain IIA. In contrast, the primary binding site for hemin is located in domain I [7,8].

Meloxicam [4-hydroxy-2-methyl-*N*-(5-methyl-2-thiazolyl)-*2H*-1,2-benzothiazine-3-carbo-xamide-1,1-dioxide] ($C_{14}H_{13}N_3O_4S_2$) (Scheme 1) is pharmacologically important new generation, non-steroidal anti-inflammatory drug (NSAID) of enolic acid class compounds with minimum adverse gastrointestinal and renal side effects associated with traditional NSAID [9]. The inhibition of the prostaglandin synthesis through the blockade of cyclooxygenase (COX) has been widely accepted as the mechanism of action of these compounds, the so-called coxibs. Meloxicam makes selective inhibition to COX-2 more than COX-1 [10,11]. The primary function of these drugs is anti-inflammatory effect but they can also be used as agents in cancer treatment, because in various types of

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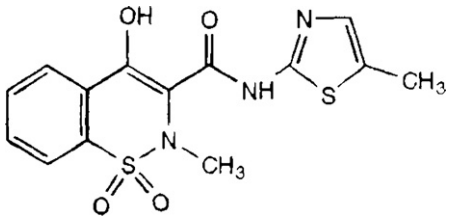

Scheme 1. The chemical structure of meloxicam.

cancer, COX-2 is over expressed [12]. COX-derived prostaglandins (PGs) have been shown to modulate cell proliferation, apoptosis, angiogenesis, and immune surveillance [13].

The plasma protein binding of meloxicam is more than 99% [14]. Meloxicam exhibits a high degree of binding to albumin, a low apparent volume of distribution and a long plasma half-life (>20 h) [15,16]. The binding of coxibs to blood components has been investigated by Seedher and Bhatia [17,18]. The nature of interaction of meloxicam with HSA involve strong drug–protein interactions with only one high affinity site located in subdomain IIA, with association constants of the order of 10^5. The binding involves predominantly hydrophobic interactions although hydrogen bonds cannot be excluded. In earlier investigation of new generation, non-steroidal anti-inflammatory drug, the precise binding site on HSA molecule, comparison with the traditional NSAID binding site and the endogenous ligands has not been determined. The competition between two drugs for their binding to albumin can significantly affect the disposition of both drugs, with possibly serious physiological consequences. These phenomena are very important if the drug is tightly bound (over 90%) to HSA. Most non-steroidal anti-inflammatory drugs show a high degree of binding to albumin, which is a primary determinant of their pharmacokinetic properties.

In this work, changes in the albumin micro surrounding caused by meloxicam binding were studied by CD and fluorescence spectroscopy methods. The meloxicam binding site was compared with aspirin which is the most popular traditional non-steroidal anti-inflammatory drug administered together with a new generation of drugs.

2. Experimental

2.1. Materials

High purity HSA >98% (GE) and bilirubin were obtained from Fluca BioChemica. [4-hydroxy-2-methyl-*N*-(5-methyl-2-thiazolyl)-*2H*-1,2-benzothiazine-3-carbo-xamide-1,1-dioxide] (Meloxicam), 1-anilino -8-naphthalene sulfonate (ANS), 3-(α-acetonylbenzyl)-4-hydroxycoumarin (Warfarin) were supplied by Sigma. 2-acetoxybenzoic acid (Aspirin) was purchased from Ubichem. The stock solution of meloxicam was prepared by dissolving in minimal amounts of methanol, diluted with double distilled water and used in all experiments from a freshly prepared 0.2 mM solutions (the maximum methanol content did not exceed 10% v/v). In the final step a stock solution was diluted with the buffer and added drop wise to the protein solution. HSA concentration was determined by absorption spectrum, taking the absorbance of a 1 mg/cm^3 solution at 280 nm as 0.55 [19]. All procedures involving bilirubin were carried out under minimal light. The appropriate amount of the substance was dissolved in 0.1 M NaOH, rapidly diluted tenfold and used in this form within 1 h. Concentrations were determined spectrophotometrically using an absorption coefficient of 52 mM^{-1} cm^{-1} at 437 nm [19]. In all of the experiments, a sodium phosphate buffer (0.05 M, pH 7.4) containing 0.1 M NaCl was used. All solutions were prepared at pH 7.4 ± 0.1 in 0.05 M sodium phosphate buffer.

2.2. CD measurement

Circular dichroism measurements were recorded at 25 °C on a Jasco J-715 spectropolarimeter, over the range of 190–250 and 300–600 nm, using 0.1 and 1.0 cm cuvettes respectively at room temperature. The spectra are expressed as MRE (mean residue ellipticity) in deg cm^2 dmol^{-1} and Mol CD in dm^{-3} dmol^{-1} cm^{-1} respectively.

2.3. Fluorescence measurement

Emission fluorescence spectra were recorded using SLM Aminco SPF-500 spectro-fluorimeter and 1 cm × 1 cm × 4 cm quartz cells with the excitation and emission wavelength set at 335 and 378 nm (warfarin), 487 and 530 nm (bilirubin), 370 and 470 nm (ANS) respectively. Tryptophan fluorescence of HSA was measured by exciting the protein solution at 295 nm. The emission spectra were recorded from 300 to 500 nm. Blank values corresponding to the buffer were subtracted to correct for the background fluorescence.

3. Results and discussion

3.1. Analysis of fluorescence quenching of HSA in the presence of drugs

Fluorescence quenching of protein could be used to retrieve many drug–protein binding information [20]. HSA contain two types of fluorophores, i.e. tryptophanyl and tyrosyl residues. When 280 nm excitation wavelength is used, fluorescence of albumin comes from both tryptophanyl and tyrosyl residues whereas 296 nm wavelength only excites tryptophanyl residues. Intrinsic fluorescence of HSA measured by selectively exciting the tryptophan residues at 296 nm reflects changes of the microenvironment of tryptophan residue in subdomain IIA, the participation of tyrosine residues concern subdomain IIIA. The interaction of meloxicam with HSA at physiological conditions (pH 7.4) was evaluated by measuring the intrinsic fluorescence intensity of protein before and after addition of meloxicam (Fig. 1). The drug causes comparable

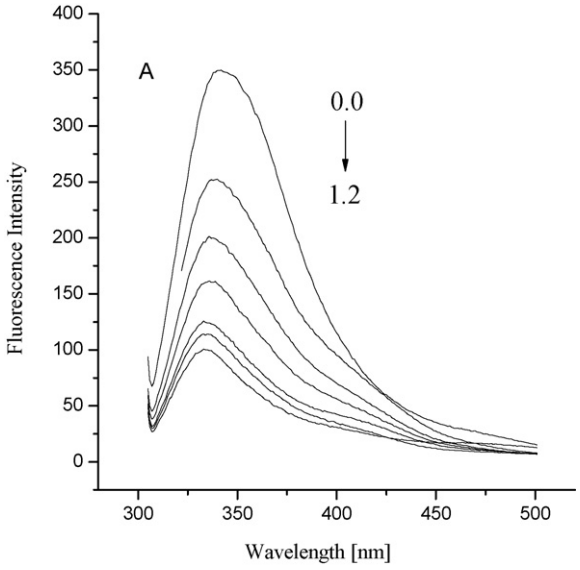

Fig. 1. Fluorescence spectral changes of HSA incubated with meloxicam at various molar ratios, for 24 h at 37 °C. Concentration of HSA: 4 × 10^{-5} M. Excitation at 295 nm. Molar ratio meloxicam/HSA: 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2.

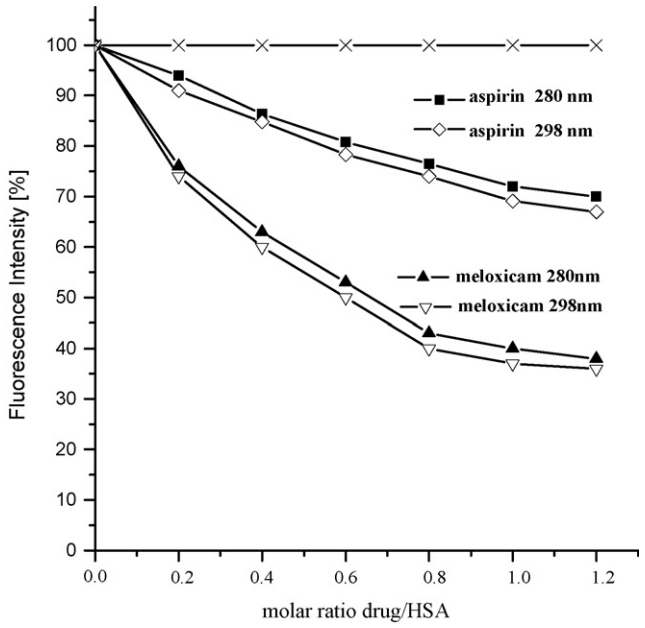

Fig. 2. Quenching of HSA fluorescence by meloxicam and aspirin under identical conditions. Excitation at 295 and 280 nm. Concentration of HSA = 4×10^{-5} M, drugs -HSA were incubated 24 h at 37 °C at molar ratio from 0.0 to 1.2. The relative fluorescence intensity of native HSA (-x-) was set as 100%.

changes in the fluorescence emission spectra at 340 nm on excitation at 280 and 298 nm, indicating that tyrosine residues are not involved in the binding. Comparison of influence of meloxicam and aspirin on the microenvironment of tryptophan residue in subdomain IIA it is shown in Fig. 2. Meloxicam could quench about 65% of HSA fluorescence, while aspirin quench only about 30%. No change of the fluorescence intensity was observed for the control HSA solution over an incubation period. In Fig. 2 it can be observed that influence of aspirin (in a range 1×10^{-5} M–5×10^{-5} M concentrations) on the microenvironment of Trp 214 residue in subdomain IIA is much smaller compared with meloxicam suggesting different binding sites of both drugs. As previously reported [21] aspirin binds with albumin in two, IIA and IIIA, subdomains. The bond of aspirin to albumin depends from concentration of drug. It is known that no aspirin–protein interaction occurs at very low drug concentration (1×10^{-7} M), whereas at higher drug contents (1×10^{-6} M–1×10^{-4} M) the aspirin anion binding (H-bonding) is mainly through the ε-amino NH_3^+ group with overall binding constant of $K = 1.4 \times 10^4\ M^{-1}$. At high drug concentrations (1-5×10^{-3} M), acetylation of Lys-199 was observed [22]. The results clearly indicate that the conformation of the hydrophobic binding pocket in subdomain IIA is affected by meloxicam and confirmed the earlier conclusion [17] that the main place of bond the meloxicam is in subdomain IIA. In studied compartment concentrations, meloxicam are considerably stronger quencher than aspirin.

3.2. Binding studies in the presence of warfarin as site I marker and hydrophobic probe ANS

The coumarinic anticoagulant, warfarin is often used as a marker substance for a specific binding site on the albumin molecule. Warfarin binds to Sudlow's site I in a pocket formed by the packing of all six helices of sub-domain IIA [6]. The interaction appears to be dominated by hydrophobic contacts, although specific electrostatic interaction is observed. In HSA molecule binding sites for ANS are in subdomain IIA and IIIA [23,24]. Comparison of the binding of both ligands (warfarin and ANS) after meloxicam-modified albumin have been commonly carried out, using the specific properties of ligands. ANS is a hydrophobic probe which does not fluoresce in the aqueous solution when it remains in a free form, but it exhibits such an ability (λem ~ 464 nm) when it is bound to the hydrophobic surface. Warfarin has a weak fluorescence at 378 nm when excited at 335 nm, and the addition of HSA induced an increase in fluorescence intensity when warfarin binds to a single site in the protein [25]. The effect was quantitatively studied by determining the percentage of inhibition of bond ANS and warfarin after substitution albumin by meloxicam or aspirin. The percentage of inhibition was calculated using the relationship, $(F–F_{drug})/F \cdot 100\%$ [% inhibition], where F and F_{drug} are the fluorescence intensities of HSA-ligand in the absence and presence of drugs, respectively. The percentage inhibition has been plotted against the molar ratio drug/HSA (Fig. 3A, B). The strong inhibition of warfarin bound to protein after meloxicam modification (about 60% when compared to native protein) confirms that this drug bound to albumin distinctly modifies the strong binding site of warfarin. The effect of aspirin on warfarin binding site is insignificant (about 5% when compared to native protein) suggesting different places of bond of both drugs. The percentage of ANS inhibition at different drugs: protein ratios are shown in Fig. 3B. The percentage quenching was about 50% in the case of meloxicam and 18% in the case of aspirin. The binding site of ANS can be located in subdomain IIA or IIIA; it does not show large selectivity in binding to albumin. A stronger inhibition of ANS binding after aspirin modification compared with warfarin may result from the fact that aspirin show nearly equal distributions between binding sites located in IIA and IIIA subdomains. Obtained results clearly indicated that meloxicam induces significant structural changes in the three-dimensional conformation of albumin and local perturbations at the warfarin and probably many other drugs binding site of HSA.

3.3. Effect of drug binding on the affinity of HSA toward heme and bilirubin

The heme and bilirubin–albumin complexes appear as an intermediate in the plasma heme and bilirubin degradation processes. Heme is bound primarily in the HSA subdomain IB within a narrow D-shaped hydrophobic cavity, where the Tyr161 residue supplies donor oxygen for the ferric heme–iron atom [26]. Based on the CD spectra of the heme–HSA (not shown) can be concluded that meloxicam binding to HSA induces small conformational change in the heme binding site, only at large excess of drug (molar ratio drug/HSA, 10). The differences observed could be related to rearrangements of the protein conformation, on result of bond drug in different sites of albumin. To obtain information about the structural integrity, CD measurements in the far-UV region (Fig. 4) were performed. CD spectra of albumin exhibit two negative bands in the ultraviolet region at 209 and 222 nm characteristic for an α-helical structure of protein. The binding of meloxicam to HSA reduces lightly both of these bands. The slight structural flexibility which is indicated by the CD measurements is likely to change in the hemin binding site. These conclusions are in accordance with previous structural studies, that the conformational adaptability of HSA involves more than the immediate vicinity of the binding site(s) [27]. Other endogenous ligand transported through albumin is bilirubin. Several lines of evidence suggest that high affinity bilirubin binding site on HSA is located in the protein interior corresponding to a hydrophobic pocket in subdomain IIA and several drugs have a strong displacing effect on bilirubin binding. As revealed by circular dichroism spectroscopy that bilirubin binds to HSA in a dissymmetric folded ridge–tile conformation [28]. In this conformation, the bound pigment shows bisignate CD Cotton effects and exhibits greatly increased fluorescence [29]. The results shown in Fig. 5 indicate that meloxicam bound to HSA distinctly modifies the strong binding sites of bilirubin. The magnitude of Cot-

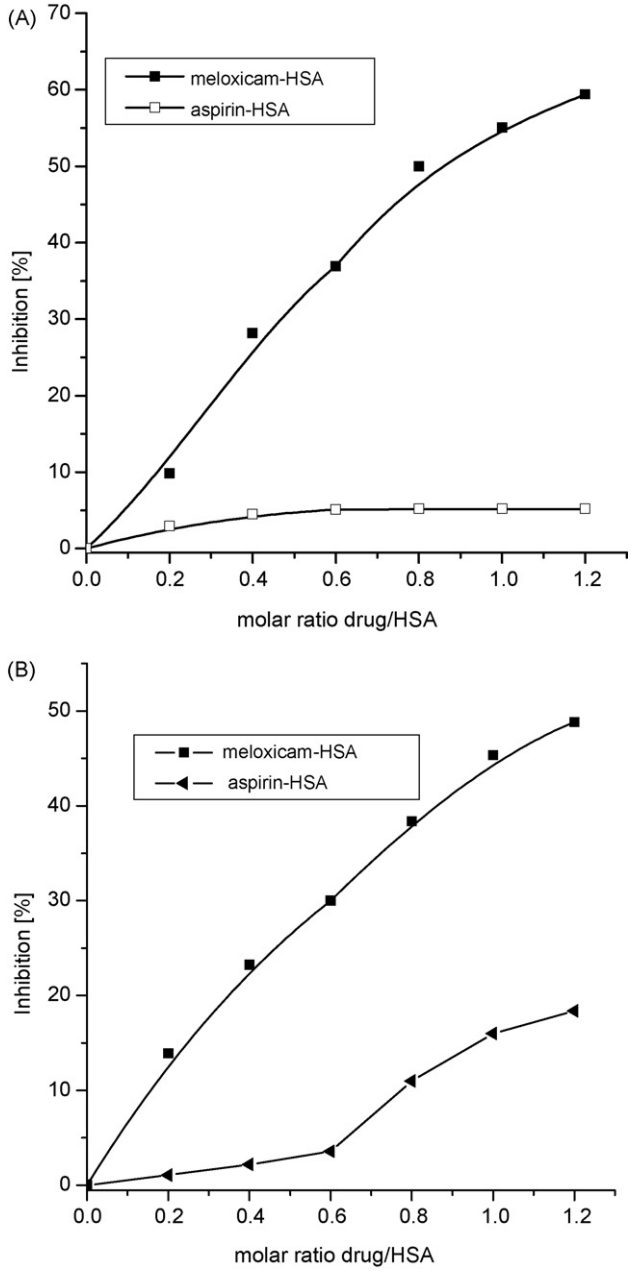

Fig. 3. The comparison of meloxicam and aspirin binding by albumin. Effect of meloxicam and aspirin on fuorescence of warfarin (A) and hydrophobic probe ANS (B) bound to albumin. Percentage inhibition has been plotted against the molar ratio drugs/HSA. Excitation wavelength, 335 nm (A) and 370 nm (B). Meloxicam and aspirin were incubated 24 h at 37 °C with HSA, before addition of warfarin and ANS. The protein concentrations were 4×10^{-5} M, the molar ratio of warfarin/HSA and ANS/HSA, 1.

ton effect of the bilirubin with meloxicam modified-HSA decreased significantly (more than 40%) when compared to native protein, during when aspirin modified-HSA decreased less than 15%. These changes in the CD spectrum of bound bilirubin have been attributed to changes in the conformation of bilirubin binding site resulting from structural changes induced in protein by drugs. These observations were confirmed by a fluorescence enhancement technique. Bilirubin–albumin solution gave an emission spectrum in the wavelength range 500–600 nm with emission maxima at 540 nm when excited at 487 nm (Fig. 5B). The decrease of the fluorescence intensity of the bilirubin bound with meloxicam modified-HSA when compared to native protein confirms that meloxicam bound

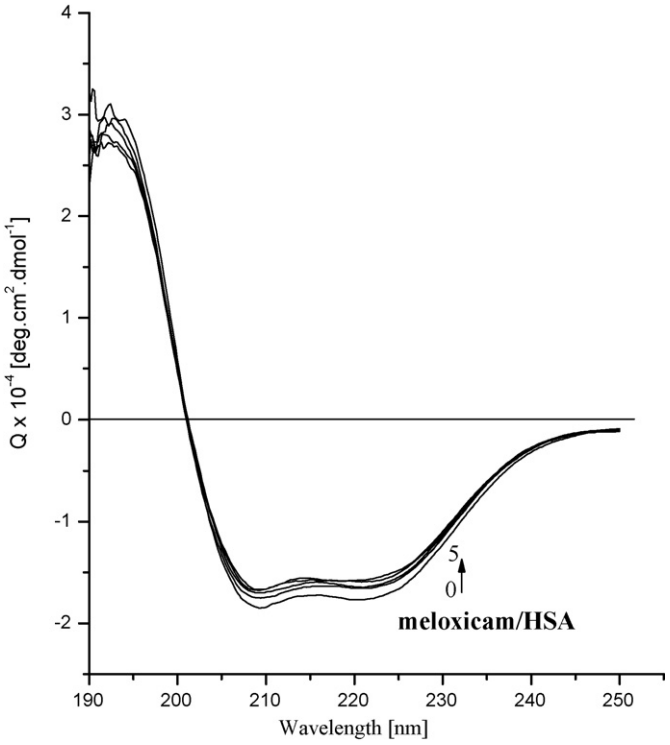

Fig. 4. CD spectra of HSA incubated for 24 h at 37 °C with meloxicam at various ratios. Molar ratio meloxicam/HSA: 0, 1:1, 2:1, 3:1, 5:1.
Far UV CD spectra were taken at protein concentrations 8×10^{-6} M with a 1-mm path length cell. The results were expressed as MRE (mean residue ellipticity) in deg cm^2 dmol^{-1}.

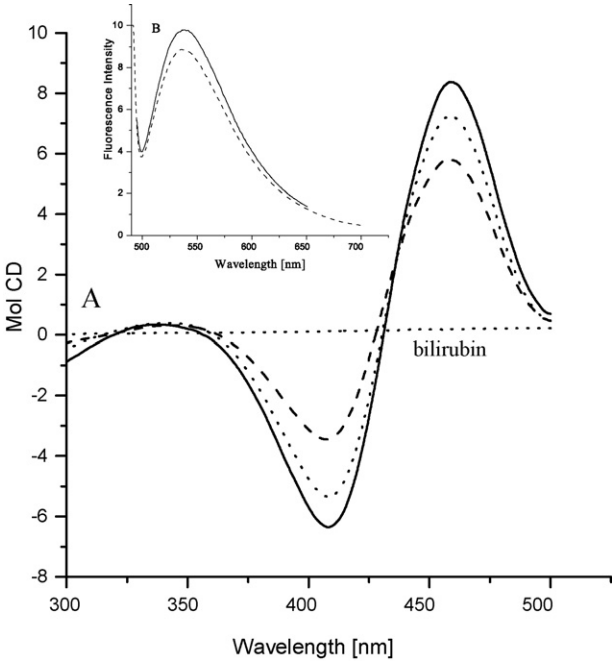

Fig. 5. Effect of meloxicam (—-) and aspirin (·····) on the visible CD spectrum of HSA–bilirubin (—) complex. Molar ratio of bilirubin/protein, 1. Concentration of HSA, 8×10^{-5} M; drugs–HSA were incubated 24 h at 37 °C at molar ratio 1:1. Mol CD units are dm^{-3} dmol^{-1} cm^{-1} (A)
Change in the fluorescence intensity of bilirubin bound to meloxicam - modified HSA. Molar ratio of bilirubin/protein, 1. Concentration of HSA, 4×10^{-5} M. Meloxicam was incubated with HSA 24 h at 37 °C, molar ratio meloxicam/HSA, 1:1. The spectra were recorded in the wavelength range 500–650 nm after exciting bilirubin–HSA (—) and bilirubin–meloxicam/HSA (- - -) complexes at 468 nm. (B).

to albumin distinctly modifies the strong binding site of bilirubin.

The obtained results suggest that the modification of albumin by new generation, non-steroidal anti-inflammatory drug meloxicam affect greatly the affinity to the primary binding sites (Sudlow site I) of albumin, particularly in the area of the binding of warfarin. The differences observed could be related to rearrangements of the protein conformation, regarding both the secondary structure and the local conformation.

4. Conclusions

The competition between two drugs for their binding to albumin can significantly affect the disposition of both drugs, with possibly serious physiological consequences. These phenomena are very important if the drug is tightly bound (over 90%) to HSA. Most non-steroidal anti-inflammatory drugs show a high degree of binding to albumin, which is a primary determinant of their pharmacokinetic properties. The information about binding of meloxicam and aspirin to albumin and the location of binding site of both drugs seems to be very important because meloxicam can be used together with aspirin, which is not only the most popular traditional anti-inflammatory drug but also anticoagulant used in treatment and prevention of cardiovascular disorders. The interaction of meloxicam with HSA causes: a conformational change with the loss of helical stability of the protein; the strong quenching of the Trp 214 fluorescence indicating that the conformational change of the hydrophobic binding pocket in subdomain IIA takes place and a local perturbation of the warfarin binding site. The strong inhibition of warfarin bound to protein after meloxicam binding (about 60% when compared to native protein) clearly indicates a significant modification of warfarin binding site. This place is also a place of binding of many other drugs including azapropazone, phenylbutazone and some sulfonamides. The affect of aspirin on warfarin binding site is insignificant (about 5% when compared to native protein) suggesting different places of bond of both drugs. Based on fluorescence and CD spectral results it can be concluded that binding of meloxicam to HSA competes with protein–bilirubin interactions. It is difficult to determine whether modification of the binding site of bilirubin is caused by conformational changes in the albumin molecule (allosteric effect), whether through direct occupation of the place by meloxicam.

Site I in subdomain IIA seems to be large and flexible and to have a large number of individual ligand-binding sites that sometimes are independent of each other but in other cases influence each other mutually. The presented results indicate that binding sites of meloxicam and aspirin though they are in the same subdomain do not overlap.

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