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THE FLUORESCENCE SIGNAL OF ALBUMIN-BOUND BILIRUBIN TO MONITOR DRUG-INDUCED BILIRUBIN DISPLACEMENT FROM HUMAN SERUM ALBUMIN

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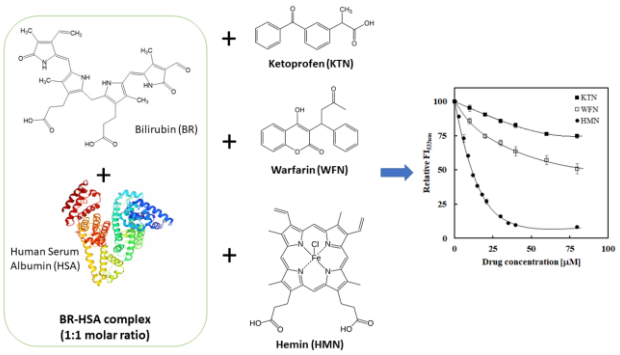
Bilirubin (BR) binding to human serum albumin (HSA) may be affected by drugs, which may cause hyperbilirubinemia. A new method is proposed to investigate BR displacement from HSA using the characteristic fluorescence signal of albumin-bound BR at 533 nm upon 460 nm excitation. Any decrease in the fluorescence intensity at 533 nm upon drug addition is direct evidence of BR displacement. Drug-induced BR displacement was tested using the well-known drug markers, warfarin (WFN) for Site I, ketoprofen (KTN) for Site II and hemin (HMN) for Site III of HSA. The maximum quenching (93%) and the highest K_a value (1.13×10^5 M^{-1}), obtained with HMN, suggested a common binding site of BR and HMN. Relatively lower BR displacement by WFN and KTN and lower K_a values can be attributed to allosteric phenomena. These results showed the importance of the albumin-bound BR fluorescence signal in testing BR displacement from albumin.



INTRODUCTION

The catabolism of hemoglobin in the bloodstream produces a toxic yellow pigment, bilirubin (BR), which is transported to the liver through binding to human serum albumin (HSA). Glucuronosyl transferase catalyzes the conjugation of BR with glucuronic acid to form conjugated BR, which is water-soluble and excreted from the body in the form of bile.[1,2] The BR binding to albumin is rapid, reversible, and robust. The association constant ranges from 6.5×10^6 M^{-1} to $>10^8$ M^{-1} due to various factors such as competitive or allosteric phenomena, buffer composition or assay methods.[3–6]

Many drugs are known to cause neonatal jaundice, kernicterus, or severe brain damage when administered to newborn infants with unconjugated hyperbilirubinemia.[6–8] One of the risk factors of neonatal jaundice is the binding of drugs to HSA at the same BR binding site, leading to the release of

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free BR into the serum.[6] BR contains two carboxylic acid groups with several polar functional groups and is characterized as a highly lipophilic compound.[9] Thus, excess free BR accumulates in the tissues and may go to basal ganglia and brain stem nuclei after it enters the central nervous system, causing severe neurological damage and kernicterus or various delayed manifestations of brain damage.[10–12] Nevertheless, BR with appropriate concentration possesses an antioxidant property in extracellular fluids.[10] Its antioxidant capacity is more substantial than superoxide dismutase, catalase, vitamin C, and vitamin E (α-tocopherol).[13]

The three-dimensional structure of HSA comprises domains I, II, and III. Each domain consists of subdomains A and B. BR's primary and secondary binding sites are centred at Site III and Site I, in subdomains IB and IIA of HSA, respectively.[14,15] Upon binding to albumin, a non-fluorescent BR produces fluorescence when excited at 460 nm.[5,16] BR-HSA complex shows the same fluorescence signal, while both free BR and HSA do not produce any fluorescence signals upon this excitation.[5,16] Although methods such as the peroxidase method, equilibrium dialysis and spectrophotometry are available to study drug-induced BR displacement, they are time-consuming or complex to standardize.[17] Hence, the present study used albumin-bound BR fluorescence as a probe to determine the drug-induced BR displacement. The three marker drugs for Site I, Site II, and Site III used in this project were warfarin (WFN), ketoprofen (KTN), and hemin (HMN), respectively. Fluorescence quenching titrations of the BR-HSA complex were conducted to determine the BR displacing action of these drugs by monitoring the reduction in the fluorescence intensity of the BR-HSA complex at 533 nm (FI_{533nm}) when site marker drugs were added at increasing concentrations. The findings may be helpful to study the BR displacing potential of any new drug used to treat other ailments.

RESULTS AND DISCUSSION

The BR-HSA complex produced a strong emission at 480–600 nm with λ_{em} of 533 nm, whilst the spectra of BR (spectrum 'a'), HSA (spectrum 'b'), WFN (spectrum 'c') and WFN-HSA mixture (spectrum 'd') produced insignificant fluorescence signals within this range (Fig. 1). Thus, a decrease in the fluorescence intensity (FI_{533nm}) in the drug's presence reflected the displacement of BR from the BR-HSA complex. As evident from Fig. 1, the addition of increasing concentrations (10–80 μM) of Site I drug WFN in the incubation mixture led to a gradual reduction in the FI_{533nm} value and blue shift in the λ_{em} of BR-HSA complex. The presence of 80 μM WFN in the reaction mixture lowered the FI_{533nm} of the BR-HSA complex by 50%, accompanied by a blue shift of 10 nm. These changes in the fluorescence characteristics indicated microenvironmental variations of the bound BR upon drug addition.[18] The binding of WFN to the BR-HSA complex reduced the energy difference between the ground and excited states to different extents, as shown by the difference in the wavelength shifts.[19] Since BR, HSA, WFN or WFN-HSA complex did not emit any significant fluorescence at this wavelength, any decrease in the fluorescence intensity can be attributed to the BR release from the BR-HSA complex.

Inclusion of the Site II drug, KTN, at increasing concentrations (10–80 μM) also produced a gradual decrease in the FI_{533nm} and a slight shift in the λ_{em} (Fig. 2), being 25% and a 2 nm red shift, respectively, at 80 μM KTN. These results did suggest BR displacement from HSA brought about by KTN. However, the displacement was modest at lower KTN concentrations and increased at higher molar concentrations. Figure 3 shows the fluorescence spectra of the BR-HSA (1:1) complex in the presence of increasing concentrations (3–80 μM) of the Site III drug, HMN. Both decline in the fluorescence intensity and red shift in the λ_{em} with increasing HMN concentrations were observed and supplementation of 3 μM HMN generated an 11% loss in the FI_{533nm} value and a red shift of 5 nm (Fig. 3).

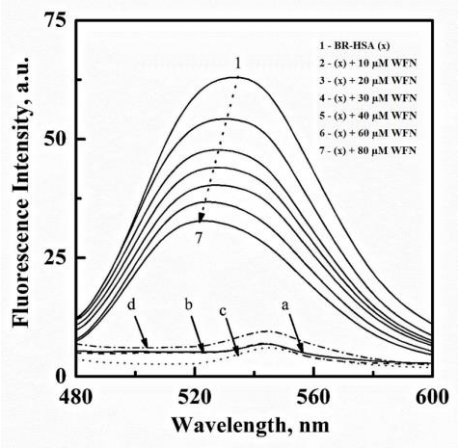

Fig. 1 – BR-HSA fluorescence spectra in the presence of increasing (10–80 μM) WFN concentrations, obtained in PB 8.0, upon excitation at 460 nm. The BR, HSA, WFN and WFN-HSA fluorescence spectra were also recorded under similar conditions and are labelled as 'a', 'b', 'c' and 'd', respectively. The concentrations of BR and HSA were fixed at 5 μM each.

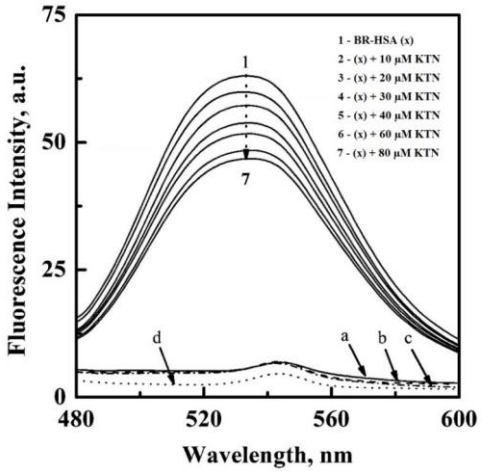

Fig. 2 – BR-HSA fluorescence spectra in the presence of increasing (10–80 μM) KTN concentrations, obtained in PB 8.0, upon excitation at 460 nm. The BR, HSA, KTN and KTN-HSA fluorescence spectra were also recorded under similar conditions and are labelled as 'a', 'b', 'c' and 'd', respectively. The concentrations of BR and HSA were fixed at 5 μM each.

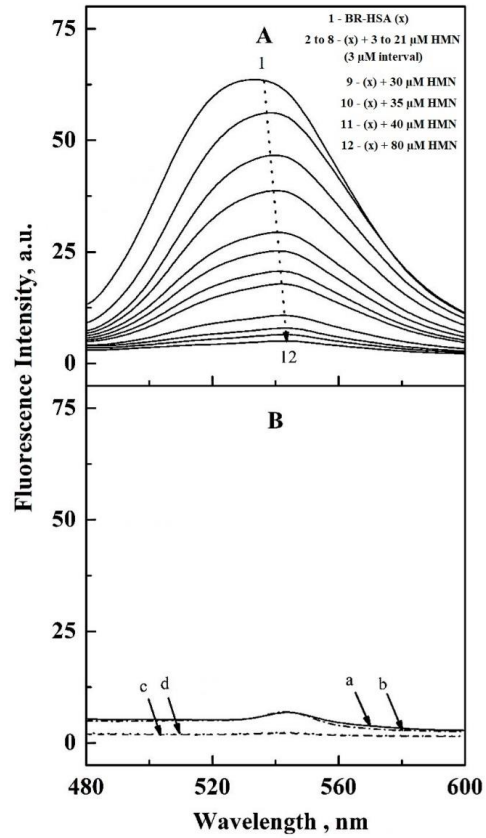

Fig. 3 – A) BR-HSA fluorescence spectra in the presence of increasing (3–80 μM) HMN concentrations, obtained in PB 8.0, upon excitation at 460 nm; B) BR, HSA, HMN and HMN-HSA fluorescence spectra were also recorded under similar conditions and are labelled as 'a', 'b', 'c' and 'd', respectively. The concentrations of BR and HSA were fixed at 5 μM each.

A comparative picture of the drug-induced quenching of albumin-bound BR fluorescence is visible in Fig. 4, where relative FI_{533nm} was plotted against drug concentration for each marker drug. KTN produced less quenching indicating a smaller amount of BR displacement; marked BR displacement was noticed with HMN. On the other hand, quenching induced by WFN was significantly greater than that observed with KTN but lower than that produced with HMN. As described above, the quenching produced by 80 μM KTN, WFN and HMN was found to be 25%, 50% and 92%, respectively, thus making HMN the most potent displacement drug used in this study.

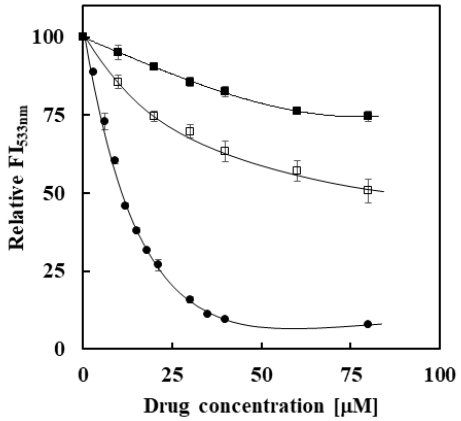

Fig. 4 – Plots showing relative FI_{533nm} of BR-HSA (1:1) complexes with increasing concentrations of marker drugs; WFN (□), KTN (■) and HMN (●), obtained in PB 8.0.

Binding affinity (K_a) values of the marker drug displacement interactions with the BR-HSA complexes were determined from the double logarithmic plots shown in Fig. 5. A higher value (1.13×10^5 M^{-1}) of K_a was obtained with HMN binding than the K_a values observed with WFN (1.26×10^4 M^{-1}) and KTN (1.24×10^4 M^{-1}) binding to the BR-HSA complexes. The significant fluorescence quenching observed with HMN indicated intense binding competition between HMN and BR for Site III of HSA. In addition, the highest K_a value obtained from HMN quenched BR-HSA complexes indicated a strong ability of HMN to displace BR binding to HSA compared to WFN and KTN binding and support the above results. A relatively higher binding affinity (K_a = 1.1×10^8 M^{-1}) than the binding affinity of BR (1.6×10^6 M^{-1}) at subdomain IB of HSA (Site III) was also reported previously, which might have contributed to the substantial BR displacement.[15,20] These results are understandable as HMN competes with BR at the same site of HSA while WFN and KTN bind to other sites of HSA.[21,24] Hemin binding to HSA has been shown to involve

a triad of basic residues Arg114, His146, and Lys140 in the hydrophobic pocket of subdomain IB and supported by Tyr161, Leu139, His146, Ile142, and Leu154 to accommodate it.[21]

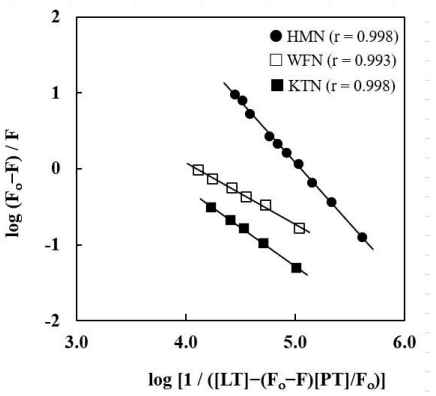

Fig. 5 – Double logarithmic plots were obtained from the data analysis of Fig. 4.

WFN produced a moderate quenching of BR-HSA fluorescence with a ~50 % decrease in FI_{533nm} at the highest (80 µM) concentration. This suggests that WFN competed partially for the BR binding site in subdomain IIA of HSA due to allosteric coupled between the group I drug with BR in the protein. However, the secondary BR binding site may not be saturated at a [BR]:[HSA] molar ratio of 1:1. The primary binding force between WFN and HSA is hydrophobic interactions as predicted from the thermodynamic results based on positive ΔH and positive ΔS values.[22] Moreover, WFN has been shown to be surrounded by uncharged residues such as Tyr150, Leu238, and Leu260 in the subdomain IIA of HSA, contributing to the hydrophobic interactions while three hydrogen bonds between WFN and Tyr150, Arg222, and His242 stabilize the WFN-HSA complex.[23] The involvement of Tyr150 in the WFN-HSA complexation might have destabilized the BR-HSA complex. Since the WFN site is adjacent to BR binding site on HSA, there are more significant conformational changes in the BR binding site upon WFN-HSA complexation.

Lower quenching of the HSA-bound BR fluorescence observed with Site II marker, KTN rendered minimal BR displacement effect, showing only 25% reduction in the $FI_{533\ nm}$ at the highest KTN concentration. The low BR displacement indicated the interaction of KTN and BR at two different binding sites of HSA, as observed earlier with the KTN and WFN binding to HSA.[24] The primary binding site of KTN at subdomain IIIA (Site II) is distinct from the BR binding sites located at subdomains 1B and IIA. Therefore, any BR displacement from its binding sites on HSA by added KTN was due to allosteric effects induced by KTN binding, which caused a conformational change in HSA rather than the competitive phenomenon. However, the secondary KTN binding site lies in subdomain IIA with a lower binding affinity (K_a = 8.99 × 10^3 M^{-1}).[18] Being adjacent to the primary BR binding site (subdomain 1B), the binding of KTN to its secondary site in subdomain IIA might have influenced the conformation of the BR binding site, resulting in its release from the binding site.

MATERIALS AND METHODS

Protein, drugs and reagents

Essentially fatty acid-free human serum albumin (Lot # SLBM7779V), bilirubin, warfarin, ketoprofen and hemin were procured from Sigma-Aldrich Co., St Louis, MO, USA. Other reagents were of analytical grade purity.

Stock solutions of protein and drugs

To prepare the protein stock solution, a fixed amount of HSA powder was dissolved in a fixed volume of sodium phosphate buffer, 60 mM, pH 8.0 (PB 8.0). The spectroscopic method was employed to determine the protein concentration (ε_m = 36500 $M^{-1}cm^{-1}$ at 280 nm)[25] on a Shimadzu UV-2450 double beam spectrophotometer.

To prepare the bilirubin (BR) solution, the desired amount of BR crystals was dissolved in 5 mL of 5 mM NaOH solution containing 1 mM EDTA in a standard flask wrapped with black paper. The solution was immediately made up to 25 mL with PB 8.0. The risk of colloid formation can be eliminated at this pH.[26] To protect the BR solution from light, it was stored in the dark at room temperature and used within 2 h to prevent degradation. The concentration of BR was determined using ε_m of 47500 $M^{-1}cm^{-1}$ at 440 nm.[27] The experiments were executed under dim light.[28]

The drugs' (WFN, KTN, and HMN) stock solutions were prepared by dissolving the fixed amount of the drug in an appropriate volume of dimethylsulfoxide (DMSO).[29] To obtain the desired drug concentration, all solutions were diluted with PB 8.0. The concentration of DMSO was < 2% (v/v) in all experiments.[30,31] WFN

concentration was determined using ε_m of 13600 $M^{-1}cm^{-1}$ at 310 nm.[32]

Spectroscopic measurements

Fluorescence spectra were measured using a Jasco spectrofluorometer (model: FP-6500) by placing the BR-HSA-drug solution into a quartz cuvette of 1 cm path length. Different parameters were fixed as: excitation and emission bandwidths at 5 and 10 nm, respectively; λ_{em} = 480 – 600 nm; λ_{ex} = 460 nm; detector voltage = 310 V; scan speed of 500 nm min^{-1} and data pitch of 1 nm.

BR displacement experiments

Fluorescence titration experiments were carried out with an equimolar (1:1) BR-HSA mixture (5 µM each) in the presence of a drug (WFN/ KTN/ HMN) to study drug-induced BR displacement. Increasing concentrations (3 – 80 µM) of the drug were added to (1:1) BR-HSA mixture in different tubes. The total volume of the solution mixture was adjusted to 6 mL with PB 8.0. The buffer was also used as a blank for this experiment. After incubation of the final mixture for 30 min at 25°C in the dark for equilibrium establishment, the spectral recording was made in dim light to prevent photo-degradation of BR. The experiments were performed in triplicate.

CONCLUSION

BR displacement from HSA in the presence of drugs was successfully monitored using albumin-bound BR fluorescence as a spectral probe. The mechanisms and degrees of displacement can be predicted by observing the fluorescence quenching. As determined from the maximum decrease in the FI_{533nm} value, the BR displacement potential of the drugs followed the pattern; HMN > WFN > KTN. The BR displacing action of site marker drugs further affirmed the location of the BR binding site around subdomain 1B. Since HMN binds to Site III, located in subdomain IB of HSA, more significant BR displacement induced by the added HMN was expected. Relatively lesser BR displacement from HSA by the Site I and Site II markers also suggested sharing the BR binding site or allosteric effect. Further studies can be conducted on other drugs to determine their BR displacing potential using albumin-bound BR fluorescence as a spectral probe.

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