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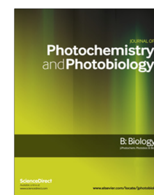

Application of phenol red as a marker ligand for bilirubin binding site at subdomain IIA on human serum albumin

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ABSTRACT

The drug–bilirubin interaction for all drugs administered especially to infants with hyperbilirubinemia should be evaluated for their ability to displace bilirubin and vice versa. In order to examine whether phenol red (PhRed) can be used as a marker for bilirubin binding site located in subdomain IIA the interaction between PhRed and human serum albumin (HSA) in buffer solution or in normal and pathological sera solutions with different HSA:bilirubin molar ratio was investigated using absorption/absorption difference spectroscopy and molecular docking method. Six sulfonamides representing the binding site in the subdomain IIA and known to influence the binding of bilirubin were used for the PhRed displacement studies. The absorption spectra for PhRed completely bound to HSA showed significant differences in the spectral characteristic relative to the spectral profile of free PhRed. The intensity of the peak originating from the bivalent anionic form of dye was strongly reduced and the maximum peak position was red-shifted by 12 nm. The binding constant (K) of the bivalent anionic form of PhRed, calculated from absorbance data, was $1.61 \cdot 10^4$ L mol^{-1}. The variations of the absorption and absorption difference spectra of PhRed in the presence of HSA–bilirubin complex were indicative of the inhibition of PhRed binding process by bilirubin. Binding of PhRed carried out in the presence of sulfonamides showed that drugs and PhRed have a common site which also involves bilirubin. In agreement with the results of the spectroscopic analysis and molecular docking it was concluded that PhRed may be applied as a marker in the study of the binding of drugs to high-affinity bilirubin binding site.

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1. Introduction

Phenol red (PhRed) is a weak organic acid and is a typical reversible pH-sensitive dye [1]. In clinical medicine it has been used for the estimation of kidney function (the test now is used only infrequently) [2], to improve accuracy in gastric secretion studies [3] and in reversible fiber-optic chemical sensors for continuous monitoring of blood pH in the patients with metabolic and respiratory problems [4,5]. Moreover, PhRed is used to detect and map the gastric distribution of *Helicobacter pylori* during endoscopy [6], as phenol red thread (PRT) test in the diagnosis of patients with ocular sicca syndrome [7] and in veterinary for measuring tear production in birds [8–10]. PhRed exhibits a structural similarity to some non-steroidal estrogens and causes significant stimulation of cells proliferation and specific protein synthesis in estrogen-responsive cells [11]. It has also a number of other applications in biochemical laboratories [12–15].

The interaction of serum albumin with various dyes such as PhRed [16–29], bromophenol blue [29–32], bromocresol green [29,33], bromocresol purple [34], indocyanine green [29], sulfobromophthalein [29] and rose bengal [29,35] was studied by numerous investigators. The results obtained by Kamisaka et al. [29] from the competitive binding of bilirubin and some dyes showed that the dyes compete for the common binding sites occupied by bilirubin, but the binding specificity and mode of interaction depend on the structure of the dyes and the site where the displacement occurs. It was found by the same authors [29] that some dyes e.g. indocyanine green and bromocresol green displaced bilirubin from its high affinity site, sulfobromophthalein had greater affinity for the second bilirubin binding site, while PhRed proved to be a weak competitor and was competitively displaced by bilirubin from albumin.

Rodkey [28] obtained evidence that albumin has two classes of binding sites for PhRed, however, a second group of sites may be occupied only at high free-PhRed concentrations. The same author stated that monoanion form of PhRed is bound about 1.5–1.7 times more strongly by human serum albumin (HSA) as is the dianion form. Kragh-Hansen et al. [17,20] who examined the binding of PhRed with HSA and relations between high-affinity binding site

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for PhRed and other ligands showed that binding of PhRed is relatively weak and at pH 7 it occurs at one high-affinity site and about five secondary sites. The studies of the binding of PhRed in pair with warfarin, digitoxin or diazepam to defatted HSA at pH 7 and at ligand/HSA molar ratios less than 1:1 showed that these ligands bind independently to the albumin at four separate primary binding sites [24]. Moreover, the investigation of Kragh-Hansen and Møller [19] indicated significant reduction of PhRed binding in the presence of bilirubin at the molar ratio bilirubin–HSA of 1:1, which meant that these substances bind to the same region on the albumin molecule. The competitive binding of PhRed to the first binding site of bilirubin was also suggested by Kamisaka et al. [29]. It was also noted that the high affinity binding site of bilirubin is not located within the azapropazone–warfarin binding region [36]. From the results cited above [21,23,26,29] it can be concluded that PhRed is located in subdomain IIA, which overlaps with the primary bilirubin binding site on HSA molecule, but not with azapropazone–warfarin binding area.

From a number of studies, it is known that certain drugs administered to the newborn infants and especially to the premature infants may compete with bilirubin for albumin binding [37–39]. The drug-induced bilirubin displacement leads to an increase in the free bilirubin (bilirubin not bound to albumin and unconjugated bilirubin) concentration and may be one of the causes of clinical risk of inducing kernicterus and neurotoxic effect of bilirubin. On the other hand, displacement of albumin-bound drugs by bilirubin is also possible and may have pharmacokinetic significance [40]. The drugs that exhibit a mutual competition with bilirubin may represent different groups of substances and they have a different degree of protein binding. Accordingly, the drugs which are bound greater than 80% by albumin interfere to a greater extent with bilirubin than substances with less than 25% binding [41]. It is difficult to predict the effect of a given substance on the displacement of bilirubin, therefore the interaction profile of bilirubin with all drugs to which neonates may be exposed should be evaluated [42,43].

The aim of this work was to show the feasibility of studying the binding of the drugs to HSA in bilirubin binding site by using PhRed as a marker of this site The sulfonamides, i.e. sulfanilamide and five N1-substituted heterocyclic sulfonamides, were selected as test drugs. These drugs represent binding site on the HSA molecule in the subdomain IIA, but the degree of binding to plasma protein (PPB%) is different in the range of 41% for sulfanilamide to 95% for sulfadimethoxine [44]. Moreover, the effect of these sulfonamides on bilirubin binding has been reported in numerous papers e.g. [40,45,46]. Numerous methods have been employed for studying the interaction of protein with drugs in vitro. Among those, equilibrium dialysis [47], ultrafiltration [47], spectrophotometry [34,48,49], electrophoresis [48] and chromatography [50] have been conventionally and most commonly used. In this work the binding of PhRed with HSA solution and sera in the absence and the presence of bilirubin and sulfonamides was studied using absorption and absorption difference spectroscopy. In order to provide further information on the specificity of the binding of PhRed with a HSA and possible interactions between PhRed, bilirubin and sulfonamides in the common binding site the computational method of molecular docking was used.

2. Experimental

2.1. Materials and solutions preparation

Phenol red (PhRed), sulfanilamide, sulfamerazine, sulfamethoxazole, sulfamethazine, sulfamethizole and sulfadimethoxine were purchased from Sigma Aldrich Chemical Co. and bilirubin was from Fluka. Human serum albumin (HSA), fraction V, crystallized and lyophilized, was obtained from Biomed (Lublin, Poland). Normal human serum (NHSerum) and pathological human serum (PHSerum) lyophilized were obtained from Alpha Diagnostics (Warsaw, Poland). The chemical structures of the sulfonamides and PhRed are shown in Fig. 1. All the experiments were performed in Sörensen's phosphate buffer (KH_2PO_4, Na_2HPO_4, 0.066 mol/L) pH 7.4. PhRed stock solution ($3 \cdot 10^{-3}$ mol/L) was prepared in the buffer and the concentration was confirmed by measuring the absorbance at 559 nm of the solution after dilution and alkalization to pH 10 so that the dye exists predominantly in its basic form and using a molar absorption coefficient $\varepsilon_{559} = 6.33 \cdot 10^4$ L mol^{-1} cm^{-1} [27]. Stock solutions of sulfonamides of 0.1–0.05 mol/L were prepared by dissolving drugs in methanol. HSA and sera were dissolved in the buffer. The concentration of HSA in the buffer was determined spectrophotometrically using a molar absorption coefficient $\varepsilon_{278} = 3.57 \cdot 10^4$ L mol^{-1} cm^{-1} [51]. Bilirubin solution at a concentration of approx. $3 \cdot 10^{-3}$ mol/L was freshly prepared by dissolving solid bilirubin in 1 mL 0.1 mol/L NaOH and adding the buffer to final solution of 10 mL. Bilirubin in the buffer solution was stirred utilizing a magnetic stirrer, in the dark at room temperature. The concentration of bilirubin was determined spectrophotometrically taking a maximum absorbance at 440 nm and molar absorption coefficient $\varepsilon_{440} = 4.72 \cdot 10^4$ L mol^{-1} cm^{-1} [52]. The aqueous solution of bilirubin was stable for about 1 h since then, due to exposure to light and air, a progressive reduction and blue-shift of the maximal absorbance occurred [52].

The stock solutions of NHSerum and PHSerum containing known amounts of proteins and bilirubin (Table 1) were obtained by dissolving lyophilized substances in the buffer. Calculations were based on the assumed molecular weight for HSA 66,500 and for bilirubin 584.7.

2.2. Spectroscopic measurements

The UV–Vis absorption spectra were recorded on Cary 50Bio UV–Visible spectrophotometer (Varian Inc.) in the range 250–650 nm, slit width of 1.5 nm and scan rate 300 nm min^{-1}. The path length of the quartz cuvette was 0.2 cm. The difference absorption spectra were obtained and analyzed using the Cary-WinUV-software vs. 3.00. All the results for the absorbance measurements presented in Figs. 2–8 are the mean of five experiments.

2.3. Ultrafiltration experiment

Ultrafiltration was performed with bags of 5 cm length made from cellulose tubing, 25 mm diameter when inflated (Dialysis Tubing Cellulose Membranes with molecular weight cut-off of 12 kDa, Sigma Aldrich Co). The bags were previously prepared as suggested by the manufacturer and then they were conditioned in the buffer for 30 min before used. Samples (3 mL) containing PhRed or PhRed with HSA were prepared by diluting the stock solutions with buffer. The bags were placed in Amicon (Merck Milipore) polypropylene caped tubes and centrifuged in MPW-223e Centrifuge (MPW, Med. Instruments, Poland) at 3000 rpm for 60 min at 23 °C. After this time, 300 μL of the filtrate, i.e. about one-tenth of the volume of solution placed inside the bag, was obtained. In all the ultrafiltrates the concentration of protein as measured by the method of Lowry et al. [53] was less than 0.1% of that inside the bag. The samples of PhRed in the buffer without albumin were used as reference solutions representing 100% of free ligand in the ultrafiltration procedure. Ultrafiltration for all the systems was performed in threefold and the average value of absorbance was used for calculation.

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