Bromocresol Green—A Hydrophobic Spectrophotometric Probe for Human Serum Albumin

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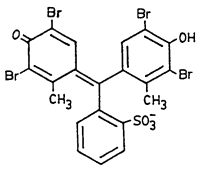
Bromocresol Green (BCG) has been developed as a new spectrophotometric probe for human serum albumin (HSA). The competing ligands naproxen (NP), 8-anilino-1-naphthalenesulfonate (ANS), and dansylsarcosine (DS) have been found to share common binding sites in HSA and their ability to displace BCG from the first competition site is in the order NP>ANS>DS.

Human serum albumin (HSA), a plasma protein, reversibly binds a variety of small molecules in the blood and transports them to their site of action. Methods such as equilibrium dialysis,[1] fluorescent probe technique,[2] circular dichroism,[3] and gel filtration[4] have been utilized to characterize the binding phenomenon. However, the versatility of the spectrophotometric technique offers advantage for carrying out the interaction studies and in the present investigation we report the applicability of Bromocresol Green (BCG) as a new spectrophotometric probe for HSA. Hitherto, only 2-(4-hydroxyphenylazo)benzoic acid has been reported as a spectrophotometric probe for BSA[5,6] for the determination of the relative affinities of drugs.

The dye, BCG, finds application[7] in the determination of albumin in the blood. The existence of BCG, at the physiological pH, in the form of nonpolar anion would be favorable for its binding onto the hydrophobic regions of HSA. This prompted us to employ BCG as a novel hydrophobic spectrophotometric probe for HSA. The ligands naproxen (NP), 8-anilino-1-naphthalenesulfonate (ANS), and dansylsarcosine (DS) are highly nonpolar and they possess structural resemblance among them. This made us to choose these three ligands as suitable competitors for BCG. BCG has been employed by us as a spectrophotometric probe for characterizing the binding of ANS and NP in BSA.[8] Hence from the competing ability of these three ligands it is our aim to determine the strength of the HSA-competitor complexes. Also this study would enlighten on the applicability of BCG as a spectrophotometric probe for HSA.



Bromocresol Green

Experimental

Materials. Human serum albumin (HSA) (fatty acid-free), 8-anilino-1-naphthalenesulfonate (ANS), and dansylsarcosine (DS) were obtained from Sigma Chemical Co., USA. Pure samples of naproxen (gift from Cipla Ltd.) and Bromocresol Green (BCG) (from Sisco-Chem, India) were used without purification. All experiments were carried out at pH 7.4 of 0.05 M phosphate buffer (1M=1 mol dm^{-3}) at 25 °C. The concentrations of HSA were determined assuming a molecular weight of 66×10^3 and checked by measuring the absorbance at 280 nm ($E^{1\%}_{1cm}$=5.3[9]).

Methods. BCG shows absorption maximum in the visible region and the binding of BCG to HSA is quantified by absorbance change method.[10] In a typical experiment, to a fixed [BCG]=28.0 μM, varying [HSA] of 1.04 to 8.36 μM were added and the absorbance of BCG and BCG-HSA mixtures were measured after 30 min at 621 nm using Carl-Zeiss Specord UV-Vis spectrophotometer. For the competition experiments, the [BCG]/[HSA] ratio was maintained at 6.7 and absorbances were measured in presence of increasing [Competitor]. The experiments were conducted in presence of NP, ANS, or DS as competitors to study their effect on binding of BCG.

Results and Discussion

Binding of BCG to HSA in Absence of Competitor. Figure 1A shows the absorbance of BCG in presence of

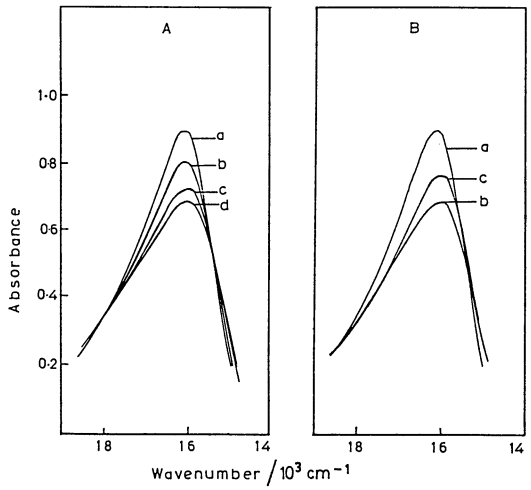


Fig. 1. (A) Absorption spectra at pH 7.4 of HSA-BCG complex. a: 24.0 μM of BCG only. b—d in presence of HSA. [HSA] (in μM)=b: 2.09; c: 4.18; d: 6.27.
(B) Absorption spectra at pH 7.4 of HSA-BCG complex in absence and in presence of ANS as competitor. a: 24.0 μM of BCG only. b: BCG+6.27 μM of HSA. c: BCG+HSA+10.0 μM of ANS.

varying [HSA]. The decrease in absorbance without change in λ_{max} evidently indicates the presence of a single spectroscopic complex. It is well-known[11] that electronic transitions of the phthaleins are susceptible to polar effects. BCG is a phthalein dye and the decrease in absorbance in presence of HSA can be attributed to the creation of nonpolar environment for the dye in HSA. An identical explanation advanced[12] for the similar spectral phenomenon for bromosulfophthalein-HSA complex supports our inference. The results of binding are analyzed by the Scatchard equation[13]

$$\frac{\bar{r}}{C_F} = nK - \bar{r}K \qquad (1)$$

where $\bar{r}$ denotes the moles of ligand bound per mole of protein, C_F is the free concentration of the ligand and nK is the total binding constant of the complex. The basic principle of the present spectrophotometric investigation is the change in the physical property, namely the absorbance of BCG by addition of increasing [HSA]. The [BCG]/[HSA] ratio employed in the study, for observing change in absorbance, varies from 3.0 to 27.0. As the change in absorbance cannot be monitored below the [BCG]/[HSA] ratio of 3.0, the $\bar{r}$ values greater than 3.5 (in the absence of NaCl) or 2.4 (in the presence of NaCl) could only be observed (Fig. 2). The linear Scatchard plots (Fig. 2) represent the presence of identical sites for BCG in HSA. The nK and n values are respectively 25.0×10^5 M^{-1} and 5.0. The corresponding values in presence of 0.2 M NaCl are found to be 4.1×10^5 M^{-1} and 9.0. As Cl^- can form ion pairs with the cationic residues of HSA, the destruction of dipolar forces between the SO_3^- group of BCG and cationic residues of HSA lead to the predominant loss in the affinity in presence of NaCl. Furthermore, as n is increased to 9, the conformational changes brought about by NaCl leading to the creation of additional sites cannot be discounted.

Effect of pH. In addition to the investigation at pH 7.4, binding of BCG to HSA has also been studied at pH 6.0 [where HSA is in N (neutral) form] and pH 8.7 [where HSA is in B (base) form] to understand the mechanism of binding of BCG. Figure 3 depicts the binding constant as a function of pH. It is discernible from the pH-profile that the affinity of BCG is higher for N than for the B form of HSA. As HSA acquires more net negative charges in the B form, effective dipolar interaction between BCG and HSA is prevented leading to the weakening of the binding. This weakening of binding in B form parallels the effect of Cl^- at pH 7.4, wherein the screening of the cationic charges in HSA by Cl^- has caused decrease in the affinity of BCG. Hence, the presence of the cationic residues in HSA is essential for the formation of dipolar interaction with BCG and consequently both hydrophobic and dipolar forces contribute to the binding of BCG.

Competition by Ligands. The spectral phenomenon occurring in presence of a competitor, ANS, has been depicted in Fig. 1B. The partial occupation of the BCG sites in HSA by the competitor results in the decrease of $D-D_1$ value (D=absorbance of unbound BCG; D_1=the absorbance of HSA-BCG complex). The decrease in $D-D_1$ value is due to the displacement of BCG by the competitor and not due to change in ε of BCG-HSA complex as proven in our previous report[14] by equilibrium dialysis.

The phenomenon of competition can be analyzed by either the method of Steinhardt et al.[15] or the modified Hill method as developed by Flanagan.[16]

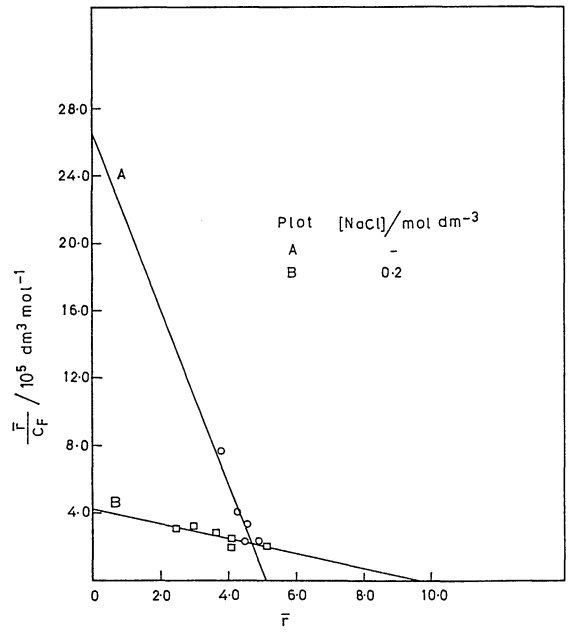


Fig. 2. Scatchard plots for the BCG-HSA interaction at pH 7.4 in the absence and in the presence of NaCl. At constant [BCG]=28.0 μM, [HSA] (in μM)=1.04 to 8.36.

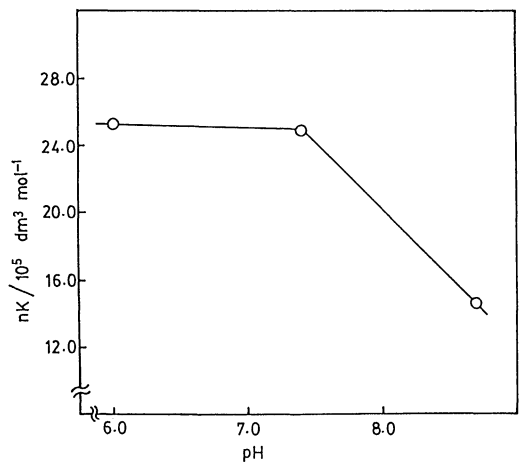


Fig. 3. pH- profile of the binding constant of BCG-HSA complex. At constant [BCG]=28.0 μM, [HSA]=1.04 to 8.36 μM at pH 6.0 and 7.4 and 1.04 to 10.45 μM at pH 8.7.

The main distinction between the two methods can be described briefly as follows. (i) In the method of Steinhardt et al., which is based on multiple equilibria, there is no requirement such as the saturation of competing sites by the probe whereas for the method of Flanagan to be applicable the essential criterion is that the competing sites should be saturated so as to conform to the following Eq. 2.

$$p_A + p_C = 1 \quad (2)$$

where p_A is the fractional saturation of HSA by the probe in presence of the competitor; p_C is the fractional saturation of the probe sites by the competitor in presence of the probe. (ii) Further, in the method of Steinhardt et al. it is assumed that there is no interaction between the bound species, namely the probe and the competitor, whereas such an assumption is not valid in the Flanagan method. Since the present study is based on the saturation of the competing sites in HSA, Flanagan method would be more suitable relative to the other method and it would be advantageous as to detect the occurring interaction between the probe and the competitor during the competition. Accordingly, for the competition phenomenon described by Eq. 3

$$PA_n + \lambda C \rightleftarrows PA_{n-\lambda}C_\lambda + \lambda A \quad (3)$$

where P, A, and C are respectively HSA, probe, and competitor; n and λ are respectively the number of sites for the probe and sites replaced, the following deduction can be made.

$$\lambda = \frac{\mathrm{d} \log [p_C/(1-p_C)]}{\mathrm{d} \log ([C]/[A])} \quad (4)$$

Hence from the Hill plot of log $[p_C/(1-p_C)]$ vs. log $([C]/[A])$, the extrapolation respectively of the slope (for $\lambda=1$) or the limiting slope (for $\lambda \neq 1$) to the abscissa gives K_C/K_A.

As BCG binds, to five sites on HSA, to ensure the saturation of competing sites, the [BCG]/[HSA] ratio employed for the competition studies is 6.7. From the Hill plot (Fig. 4) the binding constants, K_C, with respect to the first competing site for the three competitors are presented in Table 1. Earlier reports[17-19] of the binding of NP, ANS, and DS to HSA established the presence of one strong and several weak sites for each of the ligands. As the strong, high-affinity site would be occupied first (initially) it is obligatory to know the binding constant (K_C) when each of the ligands competes for the first competition site of BCG than for the subsequent sites. The K_C and K_1 (the binding constant for the high-affinity site) values agree reasonably well (Table 1) indicating that the first competition site of BCG is common to all the three ligands. Hence, the strong site (initially occupied) for the three ligands is common in HSA. This observation is different from the one made from our studies with BSA[8] using BCG as a probe, where the initially occupied sites of NP and ANS are not common, and present in distinct regions. From the interaction (indicated by λ) exerted by the competitors on BCG sites in HSA (Table 1), it can be inferred that the competing ability is found to decrease for NP and DS whereas it remains unaltered for ANS with increase in p_C. As K_C values in Table 1 signify the strength of

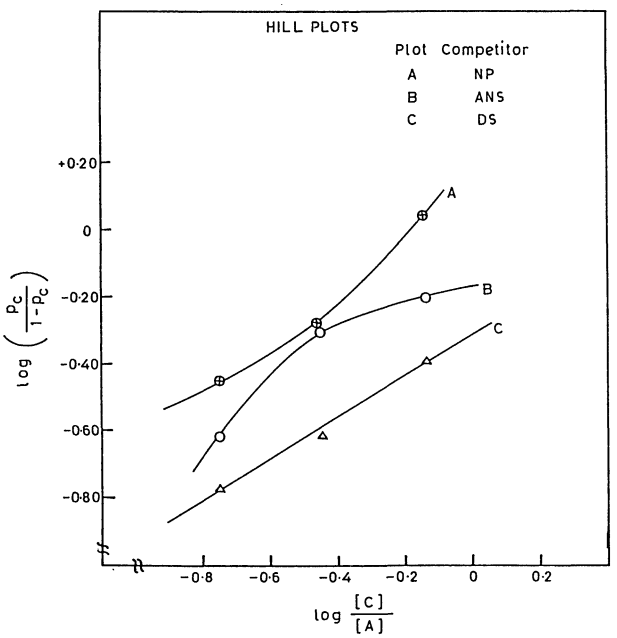


Fig. 4. Hill plots for the replacement of the BCG from HSA by the competitors NP, ANS, and DS at pH 7.4. The [BCG]=28.0 μM and [HSA]=4.18 μM such that [BCG]/[HSA]=6.7 for the saturation of the competing sites.

Table 1. Binding Paremeters for the Competitors for the BCG Sites in HSA (from Hill Plots) and Comparison with the Literature Values

Competing ligand	Interaction	$\frac{K_C}{K_A}$[a] for the first competing site	$10^{-5}K_C$ / M^{-1}	$10^{-5}K_1/M^{-1}$ (from literature)
NP	Negative	2.23	11.1	14.0[17]
ANS	No interaction	1.62	8.0	9.0[19]
DS	Negative	0.79	3.9	3.1[18]

a) K_A is $\frac{nK}{n}=4.97\times10^5$ M^{-1}.

the strong complexes of competitor-HSA, it is logical to deduce further that the displacing ability of the competitors for the first competing site of BCG is in the order NP>ANS>DS. Therefore, the present study has enabled to evaluate the strength of the binding of nonpolar ligands in HSA by utilizing BCG as a spectrophotometric probe and creates further possibility for employing BCG as a probe for varied, strongly bound, anionic nonpolar drugs and ligands.

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