Proc. Indian Acad. Sci. (Chem. Sci.), Vol. 100, No. 6, December 1988, pp. 525–533.

Bromocresol green as a new spectrophotometric probe for serum albumins

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MS received 11 January 1988; revised 16 August 1988

Abstract. Bromocresol green (BCG) has been employed as a new spectrophotometric probe to characterise the binding regions of human serum albumin (HSA) and bovine serum albumin (BSA). BCG binds with greater affinity onto BSA than onto HSA. Based on the abilities of ligands Naproxen and 1-anilino-8-naphthalenesulphonic acid (ANS) to displace BCG from the serum albumins by competitive or non-competitive mechanism, binding regions were identified for these ligands. It has been found that both Naproxen and ANS share common binding sites with BCG in HSA with the relative ability of Naproxen > ANS on binding to HSA. In the case of BSA, ANS competes with BCG for the same binding sites, whereas Naproxen exhibits non-competitive binding. The high-affinity sites of Naproxen coincide with BCG binding sites while the low-affinity sites occur at sites distinct from the BCG binding region.

Keywords. Human serum albumin; bovine serum albumin; bromocresol green; binding region; spectrophotometric probe; equilibrium dialysis; competitive displacement; non-competitive mechanism.

1. Introduction

A large number of low molecular weight compounds, including drugs, bind reversibly to human serum albumin (HSA), which functions as the major transport protein in the blood. The strength of the binding can affect the drug distribution and the intensity of drug effects.

Several methods like equilibrium dialysis (Rosenberg and Klotz 1960) and fluorescent probe techniques (Daniel and Weber 1966) have been widely used to obtain information about the strength of the binding of drugs onto serum albumin. However, the versatility of the spectrophotometric technique prompted us to apply this technique in the present study for which we made use of a new spectrophotometric probe, namely, bromocresol green (BCG). Hitherto, only 2-(4′-hydroxyphenylazo)-benzoic acid has been reported as a spectrophotometric probe for determining the relative affinities of drugs onto bovine serum albumin (Zia and Price 1975; Zia and Kamali 1976).

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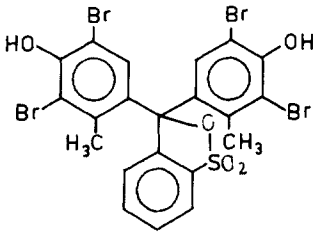
BCG has been used in the determination of serum albumin in blood (Spencer and Price 1977) and has high affinity for HSA (Bowmer and Lindup 1980). It exists in phenolic form (see structure) or in quinonoid form depending upon the pH. As the pK_a of BCG is 4·7, it exists in the quinonoid form at the experimental pH of



(bromocresol green)

Chart 1.

7·4. In the quinonoid form, a large non-polar part is connected to an anionic group which facilitates the interaction of BCG with serum albumin by hydrogen-bonding, hydrophobic and electrostatic forces resulting in its high affinity and making it a suitable spectrophotometric probe as well for serum albumins.

In the present study we have chosen a non-steroidal anti-inflammatory drug Naproxen and a non-polar ligand 1-anilino-8-naphthalenesulphonic acid (ANS) as competing ligands for BCG in serum albumins. They are found to bind strongly to HSA (Kober and Sjoeholm 1980; Sudlow *et al* 1975) and to BSA (Meenakshi Maruthamuthu and Kishore 1987b; Jun *et al* 1975). From their relative abilities to displace BCG by competitive or non-competitive mechanism, it is our aim to characterise the binding regions for the above two ligands in HSA and in BSA.

2. Experimental

Human serum albumin (HSA) and bovine serum albumin (BSA) (both are fatty acid-free), 1-anilino-8-naphthalenesulphonic acid (ANS) and the dialysis membranes were obtained from the Sigma Chemical Co., USA. Pure samples of Naproxen (gift from Cipla Ltd.) and bromocresol green (BCG) (from Sisco-Chem Industries) were used without purification. All experiments were carried out at pH 7·4 using Na_2HPO_4–KH_2PO_4 buffer of 0·05 M concentration. The concentrations of HSA and BSA were expressed on the basis of their molecular weights (69,000).

A set of typical spectrophotometric experiments of BCG binding to HSA was carried out as follows: To a fixed [BCG] = 28·0 μM, varying [HSA] were added in different sample tubes. HSA concentrations of 1·0, 2·0, 3·0, 4·0, 6·0 and 8·0 μM were used. The mixtures were maintained at 25°C for 30 min with constant shaking. Then the absorbance of BCG and BCG-HSA mixtures were measured at 621 nm using a Carl–Zeiss UV–Vis Specord spectrophotometer. It was found that 10·0 μM of HSA was required for the complete binding of 28·0 μM of BCG.

The above mentioned set of experiments were also made in presence of either Naproxen or ANS and the absorption measurements were made as before. [Naproxen] and [ANS] were varied from 5·0 to 30·0 μM to study the effect of competing ligands on BCG binding to HSA. Similarly experiments were also performed for the binding of BCG to BSA both in the absence and the presence of the competitors. [BSA] of 2·0, 4·0, 6·0, 8·0, 10·0 and 12·0 μM were used. It was

determined that 28·0 μM of BCG required 14·0 μM of BSA for complete binding. The concentrations of competitors were the same as those used in HSA–BCG experiments.

2.1 *Evaluation of the data*

If A, A_1 and A_2 denote the absorbance of BCG, respectively, in the absence, partially bound and completely bound condition to HSA or BSA, then the fraction of the dye bound to serum albumin is,

$$C_B/C_T = \frac{A - A_1}{A - A_2} = \frac{\Delta A_1}{\Delta A_2}, \tag{1}$$

where C_B is the bound concentration of BCG and C_T is the total [BCG]. From the above relation, C_B and hence r, the number of moles of BCG bound per mole of HSA or BSA can be calculated. The concentration of the free BCG, C_f, was calculated by subtraction of the bound [BCG] from the total [BCG].

When constant [ANS] or [Naproxen] was added to the albumin–BCG system, the partial occupation of the binding sites of BCG in serum albumin by the competitor leads to the displacement of BCG and hence the decrease of ΔA_1 [see (1)]. This manifests in the decrease of the fraction of BCG bound to HSA and BSA, from which C_B, C_f and r were determined for albumin–BCG in presence of varying [competitor].

The binding data obtained for the binding of BCG to serum albumins both in the absence and in the presence of the competitors were analysed according to the double-reciprocal method of Klotz (Klotz *et al* 1946),

$$\frac{1}{r} = \frac{1}{nK} \cdot \frac{1}{C_f} + \frac{1}{n}, \tag{2}$$

where nK is the total binding constant for the BCG–albumin complex and n is the number of binding sites for BCG on the albumin. From a plot of $1/r$ vs. $1/C_f$, nK and n can be determined.

When the ligand bound onto a protein is displaced by a competitor, the following relation deduced from the one proposed by Edsal and Wyman (1958) holds good:

$$\frac{1}{K_b} = \frac{1}{K_a} + \frac{[I]K_c}{K_a}, \tag{3}$$

where K_a and K_b are the total binding constants of the serum albumin–BCG complex, respectively, in the absence and presence of the competitor; K_c is the total binding constant of the albumin–competitor complex; and $[I]$ is the free concentration of the competitor. Hence $1/K_a$ and $1/K_b$ represent the slopes of the double reciprocal plots of $1/r$ vs. $1/C$, respectively, in the absence and presence of the competitor. Since it is not possible to determine the free concentration of the competitor, as an approximation $[I]_{\text{total}}$ can be used instead of $[I]_{\text{free}}$. From (3) it

can be seen that a continuous variation plot of $1/K_b$ vs. $[I]_{total}$ would result in a straight line with a positive and constant slope of K_c/K_a if the competitor competitively inhibited the binding of the probe. In such situations, where the competitors competitively inhibited the probe binding, the relative competing abilities of probes (i.e., from K_c values) can be determined. On the other hand a curve would reflect non-competitive binding.

2.2 *Equilibrium dialysis experiments*

In order to ensure that the change in physical property, i.e., absorbance of BCG–albumin, A_1, on addition of the competitor is due only to displacement of BCG, an independent technique, namely, equilibrium dialysis was carried out as mentioned in the literature (Rosenberg and Klotz 1960). For the representative experiments, [BSA] of 4·0, 6·0 and 10·0 μM were employed. The albumin solution was inside the dialysis membrane and equilibrated against 28·0 μM of BCG without and with 10·0 μM of ANS as competitor (both ligands taken outside) at 25°C for 48 h. The binding of BCG onto the membrane was found to be negligible. After equilibration, the free [BCG] outside the membrane was determined by measuring its absorbance at 621 nm. From this, C_B and hence percentage of BCG bound in the absence and in the presence of ANS were determined.

3. Results and discussion

3.1 *Binding mechanism of BCG onto HSA and BSA*

When varying [serum albumin] is added to a constant [BCG], the environment around the chromophore is perturbed resulting in the decrease of absorbance without change in λ_{max} as shown in figure 1A for the HSA–BCG system. This suggests that the displacement of monomer–polymer equilibria of the protein (Sheppard and Geddes 1945) or stacking of bound dye molecules (Bradly and Wolf 1959) is not involved in causing the observed spectral changes. This evidently also points to the presence of a single spectroscopic complex which implies that independent and identical sites were involved in the binding of BCG onto serum albumin.

Figure 2 depicts typical double-reciprocal plots for HSA–BCG without and with [Naproxen] = 5.0 μM to 20·0 μM. Linear regression analysis was adopted to determine the slope and intercept (i.e., $1/nK$ and $1/n$), from which nK and n for the binding of BCG to serum albumins were determined.

Table 1 shows that in absence of any competitor BCG binds with total binding constant $nK = 26{\cdot}3 \times 10^5\ M^{-1}$ to six binding sites (as $n = 5{\cdot}88$) onto HSA whereas it binds to three binding sites (as $n = 3{\cdot}25$) with a total binding constant $nK = 71{\cdot}4 \times 10^5\ M^{-1}$ onto BSA. It is obvious from the n values that HSA possesses twice as much capacity to bind BCG as to bind BSA. However the remarkable differences in the binding affinities can be explained by the partial involvement of BCG on binding to HSA in contrast to the participation of the whole BCG molecule on binding to BSA. This would be reflected in the higher affinity of BCG for BSA than for HSA. Muller (1978) has proposed such a

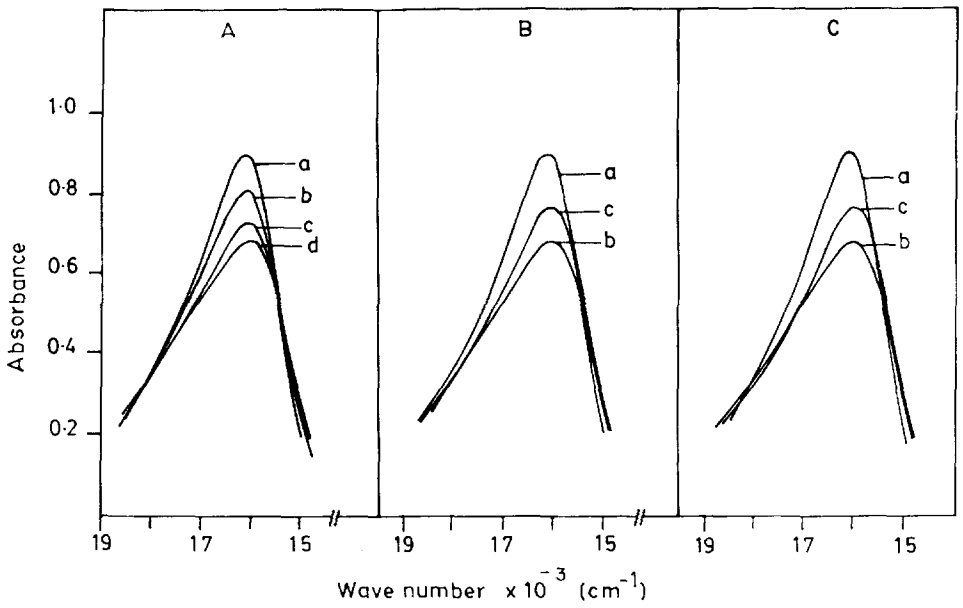


Figure 1. Absorption spectra of 20·0 μM of BCG at pH 7·4 for the HSA–BCG system. [competitor] = 10·0 μM. (**A**) free BCG and BCG + HSA, curves a, b, c and d denote [HSA] 0, 2, 4 and 6 μM, respectively. (**B**) free BCG (a), BCG + HSA (b) and BCG + HSA + ANS (c). (**C**) free BCG (a), BCG + HSA (b) and BCG + HSA + Naproxen (c).

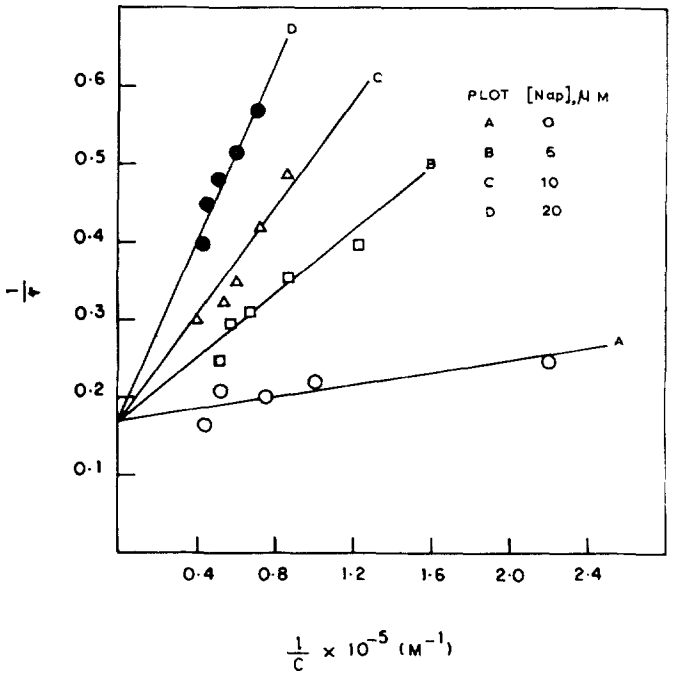


Figure 2. Klotz plots for the binding of BCG to HSA in the absence and in the presence of varying [Naproxen] as competitor.

Table 1. Binding parameters (by Klotz's method) for HSA–BCG and BSA–BCG in the absence and presence of competitors.

pH = 7·4 [BCG] = 28·0 μM
Temp. = 25°C [HSA] = 1·0 to 8·0 μM
[BSA] = 2·0 to 12·0 μM

		Competitor			
		Naproxen		ANS	
Serum albumin	[Competitor] (μM)	$\frac{1}{nK} \times 10^5$ (M)	$nK \times 10^{-5}$ (M^{-1})	$\frac{1}{nK} \times 10^5$ (M)	$nK \times 10^{-5}$ (M^{-1})
HSA	0	0·038	26·31	0·038	26·31
	5·0	0·188	5·31	0·128	7·81
	10·0	0·393	2·54	0·203	4·92
	20·0	0·690	1·44	0·360	2·77
	30·0	—	—	—	—
		n = 5·88			
BSA	0	0·014	71·42	0·014	71·42
	5·0	0·102	9·80	0·040	25·00
	10·0	0·117	8·54	—	—
	20·0	0·132	7·57	0·160	6·25
	30·0	0·150	6·66	0·240	4·16
		n = 3·25			

nK = Total binding constant; n = Number of binding sites.

possibility in explaining the higher binding affinity of intravenous biliary agents for BSA than for HSA.

3.2 *Effect of competitors on the binding of BCG to serum albumin*

Figures 1B and C show the effect of competing ligands ANS and Naproxen, respectively, on the binding of BCG to HSA. The addition of fixed [competitor] to serum albumin–BCG solution leads to the decrease of ΔA_1 [see (1)]. This can be either due to displacement or change in the extinction coefficient of bound BCG. However, equilibrium dialysis experiments revealed that at fixed [BCG] = 28·0 μM and at varying [BSA] of 4·0, 6·0 and 10·0 μM, ANS of 10 μM was able to displace, respectively, 13·6%, 13·2% and 10·8% of bound BCG. The corresponding values as obtained from spectrophotometric methods, respectively, were 14·1%, 13·0% and 11·3%. Hence the equilibrium dialysis experiment was found to corroborate the spectrophotometric method and thus confirm that the decrease in ΔA_1 value in presence of the competitor was only due to displacement of BCG.

A set of double-reciprocal plots for the displacement of BCG from HSA by varying [Naproxen] are depicted in figure 2. From this and other sets of Klotz plots (figure not shown), the slopes ($1/nK$) and total binding constant nK both in absence and in presence of competitors were obtained and are presented in table 1. The plots of $1/K_b$ vs. [Naproxen] and [ANS] for the displacement of BCG in HSA are shown in figure 3. The plots for both Naproxen and ANS gave straight lines

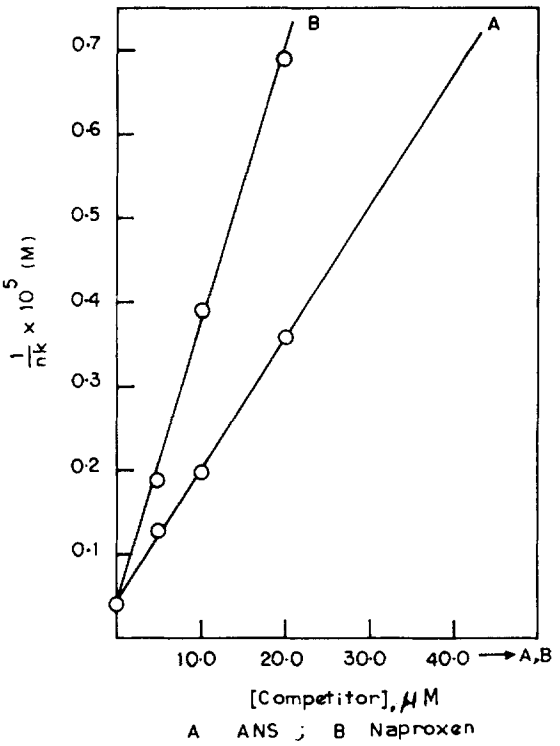


Figure 3. Plots of $1/nK$ vs. $[I]$ for the determination of competitive or non-competitive displacement by ligands in HSA–BCG.

with positive slopes for the range of [competitor] studied. As envisaged from (3), this indicates that displacement of BCG in HSA by both Naproxen and ANS occurred by a competitive mechanism. Hence the binding of Naproxen and ANS involve common binding sites in HSA. Therefore, the total binding constant K_c in HSA for the two ligands can be determined from the plot of $1/K_b$ vs. $[I]_{\text{total}}$ (figure 3). From the K_c values it was found that the ratio of K_c of Naproxen to ANS is 2·13 for the binding onto HSA which is in good agreement with the value of 1·98 as reported from independent techniques (Sjoeholm and Kober 1980; Sudlow *et al* 1975). Hence it is evident from the ratio of K_c values that the ability of Naproxen to bind to HSA is twice that of ANS.

Figure 4 represents the plots of $1/K_b$ vs. $[I]_{\text{total}}$ for the displacement of BCG from BSA by Naproxen and ANS. A straight line with a positive slope was obtained for increasing [ANS] while a biphasic curve was obtained for increasing [Naproxen]. The displacement of BCG by ANS in BSA was found to occur by a simple competitive mechanism. Considering the displacement of BCG by Naproxen it is evident from figure 4 that a steep slope was obtained initially which developed into a plateau on increasing [Naproxen]. Equation (3) reveals that the slope of the $1/K_b$ vs. $[I]$ plot would be K_c/K_a. Hence a steep slope is indicative of the high K_c value as K_a is constant. The value of K_c would be low in the region where the steep slope turns into a plateau. The steep slope was observed upto [Naproxen] = 5·0 μM. In this range, [Naproxen]/[BSA] would be low. At this ratio, Naproxen

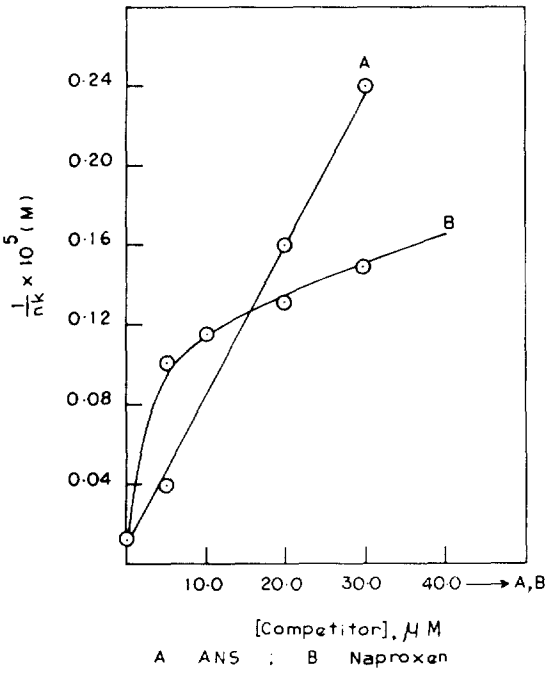


Figure 4. Plots of $1/nK$ vs. $[I]$ for the determination of competitive or non-competitive displacement by ligands in BSA–BCG.

has been found to bind only to its high-affinity site (Meenakshi and Kishore 1987). The presence of the high affinity site of Naproxen at the BCG binding region in BSA leads to the displacement of BCG as evident from the high K_c value.

At higher [Naproxen]/[BSA] ratios, the occupation of both high-affinity and low-affinity sites in BSA by Naproxen was observed (Meenakshi and Kishore 1987). The presence of the both high and low affinity sites of Naproxen in the BCG binding region would have led to the competitive displacement of BCG and would have resulted in constant K_c values for the range of [Naproxen] studied. But the reduction of the K_c value observed at the high [Naproxen]/[BSA] ratio evidently points to the presence of low-affinity sites of Naproxen in a different binding region, which is distinct from the BCG binding sites. This results in the non-competitive displacement, manifesting itself in the curvature of $1/K_b$ vs. $[I]$ plot of Naproxen. Hence, the high-affinity site of Naproxen in BSA is common with the sites possessed by BCG and ANS, whereas the low-affinity sites of Naproxen are present in a distinct binding region.

Therefore, it can be concluded that (a) BCG can be used as a spectrophotometric probe to characterise the binding regions for ligands of similar structure; (b) if the ligands are found to displace BCG by a competitive mechanism, the relative affinities of the ligands for the serum albumin can be determined on the basis of their displacing abilities; (c) if the ligand tends to displace BCG by a non-competitive phenomenon, distinct binding regions other than the probe binding sites can be identified for the ligand in serum albumin.

Acknowledgement

This work was supported by the Council for Scientific and Industrial Research, New Delhi. The authors wish to thank Cipla Ltd., India, for providing a pure sample of Naproxen.

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