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Albumin binding sites studied by high-performance liquid affinity chromatography

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Abstract

High performance liquid affinity chromatography with chiral stationary phase is used to characterize the binding sites in human serum albumin. For that purpose the possibility to use both phenylbutazone and diazepam as markers for binding sites I and II, respectively, is investigated. Their binding is characterized by affinity constants and binding capacity, determined using a mathematical method modified for more than one type of sites. The results obtained suggest that both phenylbutazone and diazepam have two types of binding sites: high- and low-affinity sites. Diazepam as an analyte binds to the high-affinity sites of the marker phenylbutazone. Phenylbutazone as an analyte binds to the low-affinity binding sites of the marker diazepam. A conclusion is derived that the binding sites for the both markers are overlapping and so they cannot be specific and entirely differentiated. Probably these are binding areas with common subsites.

Keywords: Binding sites; Markers; Phenylbutazone; Diazepam; Affinity chromatography; Serum albumin

1. Introduction

The reversible binding of many drugs to plasma proteins may often have significant effects on the overall activity profile of the compounds, including their biological distribution as well as their excretion, therapeutic activity and toxicity. Human serum albumin (HSA) is the main binding protein in serum. It was believed that there are two major binding sites on HSA (the warfarin-site and the indole-benzodiazepine-site). Sudlow et al. (1976) classified two specific binding sites on HSA, sites I and II for the anionic drugs, which corresponds to the above classification. Bruderline and Bernstein (1979) suggested that sites I and II are overlapping. Sjöholm et al. (1979) and Fehske et al. (1981) reported that two drugs, diazepam and warfarin, can be used as markers for two specific drug-binding sites, i.e. the diazepam site

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and the warfarin site, corresponding to the sites II and I, respectively. Sudlow et al. (1976) and Kurono et al. (1987) also reported that drugs binding to site II might be divided into two groups depending on whether the drug influences site I or not. A small number of drugs appear to bind to neither of these sites and so, several other minor binding sites have been proposed (Sjöholm, 1986). In recent years Carter et al. (1989) solved the three-dimensional structure of HSA and the binding loci of several organic compounds were determined crystalographically. It was found that the principal regions of ligand binding to HSA are located in the hydrophobic cavities in subdomains IIA and IIIA; warfarin occupies a single site in IIA, while diazepam has a higher degree of affinity for IIIA (He and Carter, 1992). So, the strong binding site within subdomains IIIA is commonly referred to as site II; site I corresponds to subdomain IIA.

Many experimental techniques were used to study the albumin binding of drugs. An outstanding approach appears to be the high-performance liquid affinity chromatography (HPLAC) also named biochromatography (Domenici et al., 1990; Noctor et al., 1992a; Hayball et al., 1994). In the technique proposed recently (Noctor et al., 1992b) HSA is immobilized on a suitable support—the silica template, so a chiral stationary phase (CSP) resulted. Previous studies have shown that in the case of HSA the change induced by immobilization does not affect the ligand-binding properties or its ability to modify its conformation in response to the binding of various ligands (Domenici et al., 1991). So, the investigated drug is injected onto the column and a competing agent is added to the mobile phase. The retention of a solute on the HSA columns (HSA-CSP) is clearly related to its binding affinity for the free protein. The extent of ligand-protein binding reflects in the chromatographic retention, expressed as capacity factor k'. The data obtained can be used to determine the binding affinities of different drugs, the percentage of binding, the concentration of binding sites, as well as the effects of the competitive binding. Based on that the physicochemical requirements for binding at each site can be defined and the site–site interactions on the HSA molecule can be elucidated.

Drug–drug interactions resulting in displacement of a protein bound drug by another drug (competitor) can also be studied using the HSA-CSP column, since the effect of one drug on the binding of another is immediately reflected in the change of the chromatographic retention of the tested solute. The binding of a ligand to one of the binding sites can induce a conformational change which in turn affects the binding of a second ligand to the other major binding site.

The present study concerns the possibility to use two drugs: phenylbutazone and diazepam as markers binding predominantly at each of the two major binding sites: sites I and II, respectively.

2. Materials and methods

2.1. Drugs and chemicals

Phenylbutazone (PBZ) and Diazepam (DAZ) are obtained from the National Drugs Institute (Sofia, Bulgaria). Propan-1-ol for HPLC, as well as NaH_2PO_4 and Na_2HPO_4 of purest grade are provided from Merck (Darmstadt, Germany).

2.2. Chromatography

A modular HPLC system LC-10A Shimadzu (Japan) has been used, which consists of a LC-10A pump, solvent degasser DGU-3A, Rheodyne injector with 20 μl loop, column oven CTO-10A, SPD-M10A Diode array detector and communication bus module CBM-10A. The HSA-CSP column (150×4.6 mm) is provided from Shandon Scientific (Runcorn, UK). The analysis is controled and the data are acquired with CLASS LC-10. Chromatography is carried out isocratically at temperature 34 ± 0.1°C and a flow rate 1.2 ml/min. The mobile phase is based on NaH_2PO_4-Na_2HPO_4 (67 mM, pH 7.4) modified with 8% (v/v) propan-1-ol.

2.3. HPLAC experiments

The binding of PBZ and DAZ is investigated using HSA-CSP. Two kinds of experiments are performed: (a) The drug under investigation (the

analyte) is injected onto the column; the same drug is presented in the mobile phase as a marker, i.e. the injected drug and the marker in the mobile phase are identical; (b) The injected drug (analyte) differs from the marker presented in the mobile phase. The starting hypothesis is that PBZ and warfarin bind to one and the same binding site. In order to differentiate between sites I and II binding, PBZ (instead warfarin) is first added to the mobile phase in concentrations ranging 0–30 μM. Binding at site I is affected in this way. Then the analyte (PBZ or DAZ) is injected onto the column. In the other series of experiments site II is steadily blocked adding DAZ in the mobile phase in concentrations between 0 and 40 μM. Then the analyte (PBZ or DAZ) is injected onto the column. Detection is performed at the relevant λ_{max} for the unretained substance, PBZ (264 nm) and DAZ (223 nm). The capacity factors k' are determined in duplicate for each analyte by the expression $k' = (t_R - t_0)/t_0$, where t_R and t_0 are the retention times of the analyte and the unretained compound.

2.4. Mathematical analysis

A mathematical method is used (Noctor et al., 1992a) modified for two types of binding sites (Zhivkova and Russeva, 1997).

The capacity factors k' were treated according to Eq. (1)

$$k' - X = k'_I + k'_{II} = \frac{K^I_A[S^I_{tot}]}{1 + K^I_M[M]} + \frac{K^{II}_A[S^{II}_{tot}]}{1 + K^{II}_M[M]} \quad (1)$$

concerning the case when two types of competitive binding sites exist. k' is the capacity factors at different marker concentrations [M]. k'_I and k'_{II} represent the parts of the capacity factor k' due to binding to the primary (high-affinity) and secondary (low-affinity) binding site, and K^I_A, K^{II}_A, K^I_M, K^{II}_M, $[S^I_{tot}]$ and $[S^{II}_{tot}]$ are the respective values of affinity constants of the drug, marker and common binding sites concentrations. The term X represents the part of the retention due to binding at sites where the marker does not bind. Graphical representation of Eq. (1) would be a plot consisting of two parts. The initial part of the curve corresponds to the simultaneous binding to both types of binding sites, the final part reflects only the low-affinity sites binding. For the final part of the curve of Eq. (1) looks like:

$$k'_{II} \approx k' - X = \frac{K^{II}_A[S^{II}_{tot}]}{1 + K^{II}_M[M]} \quad (2)$$

High-affinity binding in the initial phase of the curve is described by:

$$k'_I \approx k' - X - k'_{II} = \frac{K^I_A[S^I_{tot}]}{1 + K^I_M[M]} \quad (3)$$

The reciprocal of Eq. (2) and Eq. (3) yield linear relationships as follows:

$$\frac{1}{k'} = \frac{K_M}{K_A[S_{tot}]}[M] + \frac{1}{K_A[S_{tot}]} \quad (4)$$

and

$$\frac{1}{k'_I} \approx \frac{1}{k' - X - k'_{II}} = \frac{K^I_M}{K^I_A[S^I_{tot}]}[M] + \frac{1}{K^I_A[S^I_{tot}]} \quad (5)$$

K_M is determined dividing the slope $K_M/K_A[S_{tot}]$ by the intercept $1/K_A[S_{tot}]$. When the analyte and the marker are identical, there are no competition for binding sites on the column and the value of X is 0. Since $K_A = K_M$ the total concentration of binding sites $[S_{tot}]$ is easy to calculate which enables the estimation of K_A when the marker and analyte are not identical[1].

3. Results

3.1. Competitive binding studies using PBZ as a mobile phase additive (marker)

3.1.1. The same drug (PBZ) is injected onto the column as an analyte

The studies are performed by injecting small amounts of PBZ while increasing concentrations

[1] Superscripts I and II relates to the high- and low-affinity binding sites.

of PBZ as a competing agent present in the mobile phase. A decrease in the retention of PBZ is observed. The graph of $1/k'$ versus $[M]$ (Fig. 1). consists of two parts, the latter is linear and corresponds to Eq. (4). The theoretical values of k'_{II} for the initial part of the curve as well as k'_{I} (Eq. (3)) are determined. The binding parameters are estimated as it is already described according Eq. (4) and Eq. (5). The concentrations of the two types of binding sites are: $(6.40 \pm 0.22) \cdot 10^{-6}$ M for the high-affinity sites and $(6.78 \pm 1.15) \cdot 10^{-4}$ M for the low-affinity sites. The values of the respective affinity constants obtained are: $K^{I}_{M} = (1.84 \pm 0.13) \cdot 10^{5}$ M^{-1} and $K^{II}_{M} = (1.00 \pm 0.25) \cdot 10^{4}$ M^{-1}. The capacity factors k'_{I} and k'_{II} are used to obtain the retention in percentage due to the binding to the high- (15%) as well as to the low- (85%) affinity sites.

3.1.2. DAZ is injected onto the column as an analyte

The increasing concentration of the competing agent PBZ till 10 μM results in a decrease of retention, expressed by capacity factor k' (Fig. 2). Then a saturation of the sites probably is reached (at the value of $X = 3.75$), because the capacity factors are not changing further and binding to the other sites probably takes place. The affinity constants are calculated using an equation for one class of binding sites analogous of Eq. (4) and accepting a value of $X = 3.75$. A value of $K_{M} =$ $(1.72 \pm 0.60) \cdot 10^{5}$ M^{-1} is obtained for the affinity constant of the marker, which is quite identical to the high-affinity binding constant of PBZ previously defined. The common binding sites concentration already calculated is used to define the affinity constant of DAZ bound to the high-affinity PBZ sites—$K^{I}_{A} = (5.37 \pm 0.13) \cdot 10^{4}$ M^{-1}. Using $X = 3.75$, it is found that the part of the retention of DAZ due to binding to this type of binding sites of PBZ is 8%.

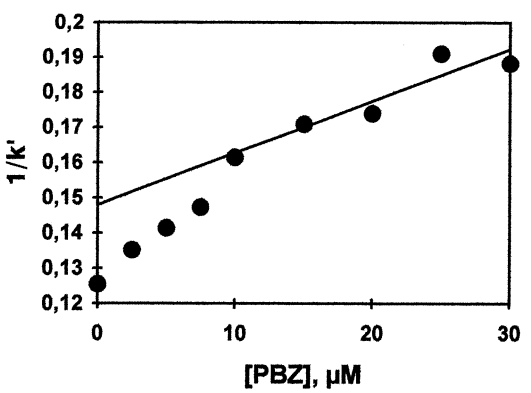

Fig. 1. Change in $1/k'$ of PBZ with mobile phase marker concentration. (●)—experimental data—bilinear behavior is obviously. (—)—theoretical plot according to Eq. (1) in the text.

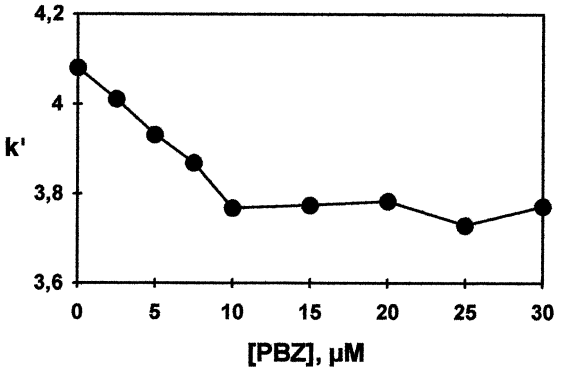

Fig. 2. Influence of the mobile phase concentration of the marker on the capacity factor of the injected DAZ. HPLC conditions: stationary phase—immobilized HSA-column 150 × 4.6 mm; eluent 67 mM phosphate buffer (pH 7.4)—propan-1-ol (92:8, v/v); column temperature 34 ± 0.1°C; flow-rate 1.2 ml/min; detection wavelengths 223 nm (for DAZ) and 320 nm (for the solvent).

3.2. Competitive binding studies using DAZ as a mobile phase additive (marker)

3.2.1. The same drug (DAZ) is injected onto the column as an analyte

Treating the capacity factors k' according to the procedure already described (Section 3.1.1), two types of binding sites are estimated as well. The affinity constants, the concentrations of binding sites and retention in percentage, due to each type of binding sites, are presented in Table 1.

3.2.2. PBZ is injected onto the column as an analyte

The experimental data $1/(k' - X)$ are plotted as a function of the marker concentrations $[M]$ in Fig. 3. The value of $X = 2$ was found by means of

Table 1
The physicochemical characteristics of diazepam-binding to HSA

Type of binding sites	Binding sites concentration, M	Association constant, M^{-1}	Part of the retention due to binding at the resp. type of binding sites, %
High-affinity	$(1.20 \pm 0.15) \cdot 10^{-6}$	$(1.00 \pm 0.18) \cdot 10^{5}$	3
Low-affinity	$(1.06 \pm 0.15) \cdot 10^{-3}$	$(4.24 \pm 0.58) \cdot 10^{3}$	97

trial and error. By further calculations, using an equation for one class of binding sites, analogous of Eq. (4), an affinity constant for the marker is obtained $K_M = (4.81 \pm 0.13) \cdot 10^3\ M^{-1}$, which corresponds well to the low-affinity constant of DAZ already found in Section 3.2.1 (Table 1). The affinity constant of PBZ to the low-affinity DAZ-binding sites is then obtained: $K_A^{II} = (6.09 \pm 0.24) \cdot 10^3\ M^{-1}$. Using $X = 2$, the part of the retention of PBZ due to binding to the low-affinity DAZ-binding sites is determined 76.3%.

4. Discussion

Albumin is the principal binding protein for the acidic drugs. It is generally accepted that there are several separate binding sites on albumin. Competition between drugs for binding sites on serum albumin is an often discussed case of drug interactions. Warfarin and PBZ are shown to bind specifically to common site: site I (Sudlow et al., 1976). Warfarin is known to be displaced by bilirubin (Roosdorp et al., 1977). Since bilirubin is not interfering with the primary binding site of DAZ (Brodersen et al., 1977) this drug must be primarily bound to a different site on the albumin than warfarin. Other authors suppose that sites for warfarin and PBZ only partially overlap and that warfarin might not be the optimal site I marker for studying HSA binding of non-steroidal anti-inflammatory drugs (Rahim and Aubry, 1995).

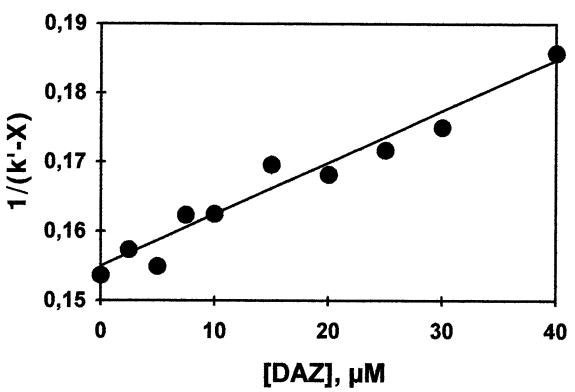

Fig. 3. Change in $1/(k' - X)$ of PBZ with mobile phase marker concentration. (●)—experimental data. (——)—theoretical plot according to Eq. (1).

The possibility to use both PBZ and DAZ as markers for different binding sites is reported in this study. The results obtained indicate that two different binding sites exist for PBZ on the HSA-CSP column: high- and low-affinity sites. It coincides well with the data reported in the literature, presented in Table 2, where different methods were used. We find that DAZ, used as an analyte, binds to the primary (high-affinity) binding sites of PBZ.

In the cases when DAZ present as a marker, high- and low-affinity binding sites are detected, as well. The low-affinity sites are shared with PBZ. The affinity constants determined in this study are in a good agreement with those found in the literature: Kurono et al. (1987) by means of fluorescent technique determined a value of $1 \cdot 10^5\ M^{-1}$; in a HPLAC study using DAZ as marker Noctor et al. (1992a) found two types of binding sites with affinity constants: $(7.2 \pm 0.5) \cdot 10^5\ M^{-1}$ and $1.9 \cdot 10^4\ M^{-1}$; almost the same value $1.4 \cdot 10^5$ gave Ascoli et al. (1995) in a circular dichroism study. Since phenylbutazone binds on the low-affinity DAZ binding sites (except on the high- and low-affinity PBZ sites) and diazepam binds to the high-affinity PBZ sites (except on the high- and low-affinity DAZ binding sites), the present results supports the hypothesis for overlapping of binding sites.

Table 2
The values of the affinity constants of PBZ as cited in the literature compared with the values estimated in the present work

Primary binding affinity constant	Secondary binding affinity constant	Method	References
$1.17 \cdot 10^5$	—	Ultrafiltration	Solomon et al., 1968
$1.00 \cdot 10^5$	$4.00.10^4$	Circular dichroism	Chignell, 1969
$2.37.10^5$	$4.56.10^4$	Circular dichroism	Rosen, 1970
$(5.06 \pm 0.86) \cdot 10^5$	$7.08 \cdot 10^3$	UV-spectroscopy[a]	Elbary et al., 1982
$(1.84 \pm 0.13) \cdot 10^5$	$(1.00 \pm 0.25) \cdot 10^4$	HPLAC	Present study

[a] Ultraviolet spectroscopy.

5. Conclusion

A conclusion may be derived from these data that the binding sites for PBZ and DAZ on the HSA-CSP column cannot be differentiated entirely. A possibility exists that they are overlapping in some extent and that common binding subsites probably present. These results confirm the concept that sites I and II are not presented as preformed sites in the albumin molecule. The sites may well be formed by extensive conformational changes during the binding process. It does not seem possible that albumin molecule has a number of localized sites for various ligands not even if overlapping is allowed. So, 'sites' could be replaced by 'binding areas' of a less specific nature, i.e. binding units which can be brought together in variable combinations according to the needs of different ligands.

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