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New mathematical approach for the evaluation of drug binding to human serum albumin by high-performance liquid affinity chromatography

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Abstract

A novel mathematical approach for investigation of drug–human serum albumin (HSA) interactions by means of high-performance liquid affinity chromatography is developed. The model is based on the assumption that two types of competitive binding sites exist on the HSA molecule. The widely used single-site binding equation is extended and a proper mathematical analysis is proposed allowing the determination of the major parameters characterizing the multisite binding (cobinding) process. The utility of the new approach is proved by competitive studies on HSA binding of two model drugs, diazepam and diclofenac. © 1998 Elsevier Science B.V.

Keywords: Drug binding; Human serum albumin

1. Introduction

Human serum albumin (HSA) is the major plasma protein responsible for the reversible binding of a wide range of drugs [1–3]. Extensive studies on different aspects of drug–HSA interactions are still in progress because of the clinical significance of the process, especially in the case of tightly bound drugs ($K_a > 10^5\ M^{-1}$) [3,4]. Numerous analytical techniques are used for protein binding studies and they are continuously being added to, along with extending knowledge about the complex mechanisms involved in the drug–HSA binding process. The advantages and limitations of the various methods are discussed by Oravkova et al. [5].

In recent years, high-performance liquid chromatography (HPLC) appears to be the optimal experimental strategy used in in vitro binding studies. Several chromatographic methods were developed for the quantitative estimation of drug–HSA interactions, including varieties of high-performance size-exclusion techniques [6–10] and high-performance liquid affinity chromatography (HPLAC) [11–18]. In the last few years, HPLAC was outlined as a 'powerful tool for the study of biomolecular interactions' [17]. In our study, HSA binding was examined in a chromatographic system consisting of immobilized HSA on a suitable support as a stationary phase and with a predominantly aqueous eluent as the mobile phase. The drug of interest (analyte) was injected onto a column and its chromatographic retention served as a quantitative measure of the binding properties. The latter could be influenced by

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adding a cobinding drug (competitor, marker) to the mobile phase.

In addition to much higher precision, reproducibility and speed, HPLAC methods ensure a very good simulation of the living biological systems since the passing of the drug through the chromatographic column occurs via a series of drug–HSA binding equilibria processes. Evidence exists that immobilization does not lead to a loss of the binding properties and the conformational mobility of the native HSA. This finding was supported by the good agreement between the data obtained by means of conventional methods and those acquired using HPLAC [11,14,15,19,20]. HPLAC was successfully applied to the resolution of a wide range of problems concerning different aspects of drug–HSA interactions, such as estimation of the equilibrium affinity constants of drugs [12,15,18], characterization of binding regions using markers for principle binding sites [13,14], elucidation of drug–drug interaction at the level of HSA binding (independent, cooperative and noncooperative binding) [13], and examination of the enantioselectivity of HSA binding [12–15,21].

The general mathematical approach is based on the relationship between the retention of the analyte in the chromatographic column (expressed by the capacity factor k') and the concentration of the marker. A simple equation has been derived for the zonal elution technique [22,23] and this has been adapted to the evaluation of drug–protein binding using HPLAC [13]. All studies to date have assumed that competition between the analyte and the marker is for one type of binding site only. Since multisite binding has also been observed, several attempts have been made to modify the single-site equations to monitor multisite or allosteric binding [13,15,17]. The usefulness of this approach is restricted to cases where at least one of the competitive drugs binds to a single type of binding site. Actually, most drugs bind to more than one type of binding site and it is quite possible that competition occurs for each of them. Results from our preliminary studies on the HSA binding of several nonsteroidal anti-inflammatory drugs (NSAIDs) to diazepam (DAZ) binding sites suggested the appearance of two types of DAZ binding site in the immobilized HSA column and competition between NSAIDs and DAZ for both of them. In this work, an expanded mathematical approach is proposed for the drug–HSA interaction that describes accurately binding and cobinding to more than one type of binding site. Results from competitive studies of DAZ and diclofenac binding are presented as an example of the usefulness of the model.

2. Mathematical approach

2.1. Competition for one type of binding site

According to the simplest model for drug–HSA binding, the drug (analyte) interacts reversibly with a single type of equivalent binding site. If a drug-marker that is competitive for the same type of binding site is added to the mobile phase, it influences the concentration of free sites and, thus, the chromatographic retention of the analyte. The capacity factor, k', declines with increasing concentrations of the marker [M] as follows [13,22,23]:

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

where K_A and K_M are the equilibrium affinity constants for the analyte and the marker, respectively, and $[S_{tot}]$[1] is the effective concentration of common binding sites. By taking the reciprocal of k', a linear plot of $1/k'$ versus [M] is obtained [13,17]:

$$\frac{1}{k'} = \frac{K_M}{K_A[S_{tot}]}[M] + \frac{1}{K_A[S_{tot}]} \quad (2)$$

The affinity constant for the marker (K_M) is obtained dividing the slope by the intercept. The concentration of common binding sites could be determined using the same substance both as a marker and as the analyte [15]. Since $K_M = K_A$, Eq. (2) reduces to

[1] In order to simplify the equations, we use the term $[S_{tot}]$ instead of m_L/V_m [13], N^0_{CA}/V_m [22] and $[L]/V_m$ [23], where m_L, N^0_{CA} and [L] represent the number of moles of common binding sites within the column and V_m is the dead volume of the column.

$$\frac{1}{k'} = \frac{1}{[S_{tot}]}[M] + \frac{1}{K_A[S_{tot}]} \quad (3)$$

and $[S_{tot}]$ is the reciprocal of the slope. The affinity constant for the analyte is further calculated using Eq. (2).

In general, the analyte not only competes with the marker for common binding sites but also binds to sites that are inaccessible to the marker. Noctor et al. [13] introduce the term X to denote the part of the capacity factor that is due to binding to these additive sites and Eq. (2) transforms to:

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

Eq. (4) is analysed as described above, the major problem being the assessment of X. Most of the authors state that it is obtained 'by iterative testing' or 'by trial and error' [13,18]. In the case of a simple single-site competition, X could be accurately defined: It is equal to k' at a marker concentration above which the value of k' remains independent of [M].

2.2. Competition for two types of binding site

Although some attempts have been made to modify the single-site equations in order to monitor multisite competition and allosteric interaction [13,15,17], they are applicable only if the analyte (or the marker) binds to a single type of binding site. Since it is believed that the HSA binding of most of the drugs, as well as the competition between them, occurs for more than one type of binding site, an extended mathematical model is necessary to describe this more complex event. In the case of competition for two types of binding site, the capacity factor, k' (measure of chromatographic retention), of the analyte consists of two terms that characterize binding to the primary (high-affinity) and to the secondary (low-affinity) binding site. Eq. (1) is modified to:

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

where k'_I and k'_{II} represent the parts of the capacity factor that are the result of binding to both common types of binding site and K^I_A, K^{II}_A, K^I_M and K^{II}_M are the affinity constants for the analyte, marker and binding site concentrations, respectively. If the additive retention (X) caused by binding of the analyte to sites inaccessible for the marker is also taken into account, Eq. (5) can be transformed to:

$$k' - X = k'_I + k'_{II} = \frac{K^I_A[S^I_{tot}]}{1 + K^I_M[M]} + \frac{K^{II}_A[S^{II}_{tot}]}{1 + K^{II}_M[M]} \quad (6)$$

Two-phase binding behavior is proposed. Initially, at lower marker concentrations, competitive binding occurs to both high- and low-affinity binding sites. After saturation of the high-affinity sites (generally poorly presented), competition continues exclusively for the low-affinity sites. For this final phase, Eq. (6) is reduced to:

$$k'_{II} \approx k' - X = \frac{K^{II}_A[S^{II}_{tot}]}{1 + K^{II}_M[M]} \quad (7)$$

The reciprocal of Eq. (7) is analogous to Eqs. (2) and (4) and all parameters characterising the secondary (low affinity) HSA binding are calculated as described in Section 2.1. The major problem is the valid evaluation of X, since, generally, the low-affinity binding sites exist at concentrations that are too high to be saturated under chromatographic conditions. X could be estimated theoretically, making an assumption that saturation of the binding sites is achieved at a marker concentration equal to the preliminary calculated low-affinity binding site concentration.

Using Eq. (7), the theoretical values of k'_{II} for the initial phase of HSA binding can also be calculated. The part of the retention due to binding to the high-affinity sites, evaluated by k'_I, is obtained by subtracting k'_{II} from k'. A similar idea has been referred to by Hage et al. [15], but mathematical treatment was not proposed.

$$k'_I \approx k' - X - k'_{II} = \frac{K^I_A[S^I_{tot}]}{1 + K^I_M[M]} \quad (8)$$

The reciprocal of k'_I yields a linear plot and all parameters of the high-affinity HSA binding are calculated as previously described.

3. Experimental

3.1. Chemicals

Diazepam (DAZ) and diclofenac (DCL) were kindly supplied by NDI (Sofia, Bulgaria). Propan-1-ol for HPLC as well as sodium dihydrogen phosphate and disodium hydrogenphosphate of the purest grade were provided by Merck (Darmstadt, Germany). The immobilized HSA column (150×4.6 mm) was obtained from Shandon Scientific (Runcorn, UK).

3.2. Apparatus

A modular HPLC system (LC-10A) from Shimadzu (Japan) was used and this consisted of an LC-10A pump, a solvent degasser DGU-3A, a Rheodyne injector with a 20-μl loop, a column oven CTO-10A, an SPD-M10A diode array detector and a communication bus module CBM-10A. Analyses were controlled and the data were acquired with CLASS LC-10 software.

3.3. Chromatography

Chromatography was carried out isocratically at a temperature of 34±0.1°C and a flow-rate of 1.2 ml/min. The mobile phase was based on NaH_2PO_4–Na_2HPO_4 (67 m*M*, pH 7.4), modified with 8% (v/v) propan-1-ol. DAZ was added to the mobile phase as the specific marker for binding site II on HSA. The required quantity of DAZ was first dissolved in propan-1-ol before addition to the mobile phase. Detection was performed at the relevant wavelength, λ_{max}; 223 nm for DAZ, 264 nm for DCL and 320 nm for the solvent, which was regarded as an unretained substance.

The competition studies were performed on the HSA column by applying mobile phases with different DAZ concentrations (0–40 μ*M*) while small quantities of DAZ or DCL were injected onto the column. The capacity factor k' was determined in duplicate for each injected compound using 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, respectively. The effects of increasing the concentration of DAZ on the retention behavior of both DAZ and DCL were analyzed according to the mathematical approach described in Section 2.

4. Results

4.1. Binding studies using diazepam as marker as well as analyte

Initial studies were performed by injecting a small amount of DAZ (1.72 nmol) onto the immobilized HSA column, while continuously increasing the concentration of DAZ (as a marker for binding site II) in the mobile phase. The capacity factors obtained are shown in Table 1. A decrease in retention was observed for the whole range of DAZ concentrations, without saturation of the binding sites. Since the marker and the analyte were identical, it could be supposed that they share only common binding sites in the column and the value of X is zero.

The corresponding graph of the reciprocal value of the capacity factor versus the DAZ concentration is presented in Fig. 1. The biphasic dependence of $1/k'$ on [DAZ] is an indication of the existence of two different types of binding site for this drug in the column. The experimental data for the final part of the curve were fitted to a linear plot and the concentration of the low-affinity DAZ binding sites as well as the affinity constant were estimated according to Eq. (3). After the theoretical values of k'_{II} and k'_{I} for the initial part of the graph were

Table 1
Influence of the mobile phase concentration of DAZ on the capacity factor of injected DAZ (k'_{DAZ}) and DCL (k'_{DCL})

[DAZ] (μ*M*)	k'_{DAZ}	k'_{DCL}
0.0	4.593	26.275
1.0	–	26.063
2.0	–	25.882
2.5	4.541	25.731
4.0	–	25.523
5.0	4.468	23.788
7.5	4.413	24.697
10.0	4.361	24.292
15.0	4.241	23.058
20.0	4.075	22.788
25.0	4.074	23.334
30.0	3.972	22.730
40.0	3.824	21.123

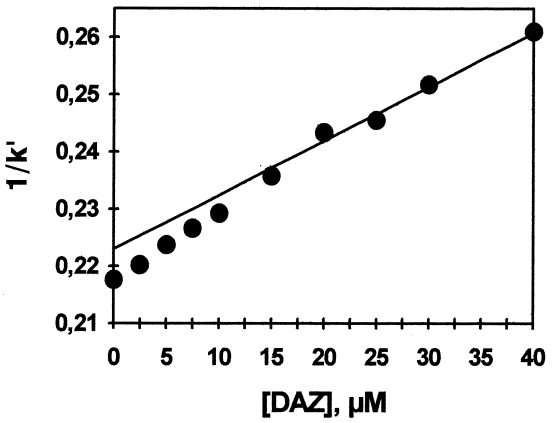

Fig. 1. Change in $1/k'$ of DAZ with the concentration of the mobile phase marker. (●) Experimental data (biphasic behavior is obvious). (—) Theoretical plot based on Eq. (3), describing the binding to the low-affinity DAZ binding sites. The best-fit line for the final phase of the curve is $y=(0.223\pm0.003)+(944.595\pm116.651)x$, with a correlation coefficient of 0.978 ($n=5$).

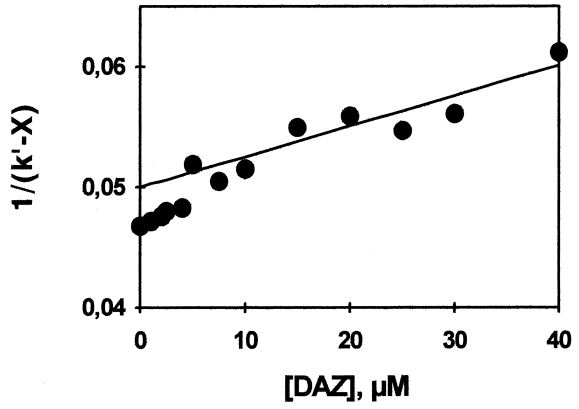

Fig. 2. Change in $1/(k'-X)$ of DCL with the concentration of the mobile phase marker. (●) Experimental data (biphasic behavior is obvious). (—) Theoretical plot according to Eq. (4), describing cobinding to the low-affinity DAZ binding site. The best-fit line for the final phase of the curve is $y=(0.050\pm0.001)+(253.455\pm52088)x$, with a correlation coefficient of 0.893 ($n=8$). The value of X is 4.89.

obtained as it was already described, the high-affinity DAZ binding sites concentration and the respective binding constant were also calculated using Eq. (2). The part of the chromatographic retention due to DAZ binding to both types of binding site was evaluated as a ratio of k'_{I} (or k'_{II}) to k' at marker concentration of zero. The major quantitative parameters of DAZ binding are summarized in Table 2.

4.2. Studies of diclofenac binding to DAZ sites

The proposed mathematical approach was further applied in binding studies on DCL to DAZ binding sites. A small amount of DCL (1.54 nmol) was injected onto the column and the effect of increasing the concentration of DAZ was evaluated. The capacity factor of DCL (also shown in Table 1) decreased over the entire range of DAZ concentrations in a biphasic manner, indicating that there is competition between DCL and DAZ for both high- and low-affinity binding sites. Saturation of the binding sites was not achieved, and the value of X was determined to be 4.89, as described above, assuming that the marker concentration necessary for occupation of the binding sites is equal to the relatively high concentration of low-affinity DAZ-binding sites (Table 2).

The corresponding graph of $1/(k'-X)$ versus [DAZ] is presented in Fig. 2. The experimental data for the final part of the curve were fitted to a linear plot and the affinity constants of the marker and analyte to the low-affinity DAZ binding sites (the concentration having been determined previously) were calculated using Eq. (4). After the part of the chromatographic retention resulting from the high-affinity binding was derived, the affinity constants

Table 2
Binding characteristics of DAZ to immobilized HSA

Type of binding site	Binding site concentration (M)	Association constant (M^{-1})	Part of the retention due to binding at this type of binding site (%)
High-affinity	$(1.2\pm0.2)\cdot10^{6}$	$(1.0\pm0.2)\cdot10^{5}$	3
Low-affinity	$(1.1\pm0.2)\cdot10^{3}$	$(4.2\pm0.6)\cdot10^{3}$	97

Table 3
Binding characteristics of DCL to DAZ binding sites on immobilized HSA

Type of binding site	Association constant of marker (M^{-1})	Association constant of DCL (M^{-1})	Part of the retention due to binding at this type of site (%)
High-affinity	$(0.9\pm0.1)\cdot10^5$	$(1.2\pm0.1)\cdot10^6$	5
Low-affinity	$(5.1\pm1.2)\cdot10^5$	$(1.9\pm0.1)\cdot10^4$	76

for binding of the marker and analyte to the primary DAZ binding sites were also calculated using the same equation. The major quantitative parameters of DCL binding to DAZ binding sites on HSA are listed in Table 3.

5. Discussion

The results presented in this work indicate that there are two types of binding site on HSA for DAZ; high-affinity binding sites at a very low concentration and widely represented low-affinity binding sites. The parameters obtained show good agreement with the values reported previously. Generally, the affinity constants cited for the primary DAZ binding site are in the range of $1–8\cdot10^5\ M^{-1}$ [12,24–27]. A value of $1\cdot10^5\ M^{-1}$ was determined by Kurono et al. [26] using a fluorescent technique that is quite similar to that determined by Askoli et al. [27] in a circular dichroism study, i.e. $1.4\cdot10^5\ M^{-1}$. Noctor et al. [13] used DAZ as a marker for site II in their HPLAC investigations and established that the retention of the compounds of interest (chiral and achiral benzodiazepines) was affected in a linear manner only up to a DAZ concentration of 1.3 μM. We found nearly the same value for the concentration of high-affinity DAZ binding sites (Table 2). The loss of linearity at higher concentrations could be explained by the saturation of these low concentration sites. Two types of DAZ binding site were also referred to in the above study, having affinity constants of $7.2\pm0.5\cdot10^5\ M^{-1}$ and $1.9\cdot10^4\ M^{-1}$. These values are in very good agreement with our results. The differences (less than one order of magnitude) are a consequence of the various experimental conditions used (temperature, pH, mobile phase composition).

The results obtained in our competitive study on DCL binding to DAZ binding sites suggest that competition occurs for both types of binding site. DCL binding to HSA has been a subject of several publications, but the strength and localization of this binding is still controversial. Most authors agree that two classes of binding site exist on HSA for DCL, a high-affinity (low capacity) site with an equilibrium constant in the range of $1–1.5\cdot10^5\ M^{-1}$ and a low-affinity (high capacity) site with a constant in the range of $3.05–3.71\cdot10^3\ M^{-1}$ [28,29]. Other authors suggest that there is only one type of binding site with an affinity constant of $5.5\cdot10^5\ M^{-1}$ [30] or $0.76\cdot10^4\ M^{-1}$ [31]. A possible explanation of the observed differences could be the variety of experimental techniques and conditions used. The localization of DCL binding sites is still unknown, but it is supposed that DCL binds to a comparable extent to both the I and II sites on HSA. The affinity constants obtained in our study are higher than those cited in the literature (Table 3). This is explicable in terms of our experimental model: we examined the binding of DCL not to the HSA molecule in general but to the specified DAZ binding sites.

As shown in this work, the proposed model adequately reflects the binding behavior of both of the model drugs used, DAZ and DCL. It could be concluded that this mathematical approach is a useful supplement to the HPLAC technique and enables more detailed and precise clarification of different aspects of drug–HSA interactions.

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