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Protein binding of some nonsteroidal anti-inflammatory drugs studied by high-performance liquid affinity chromatography

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Abstract

The protein binding of indomethacin, sulindak and diclofenac sodium is studied in the presence of some competitors: phenylbutazon and diazepam. A high-performance liquid affinity chromatography based on chiral stationary phases with immobilized human serum albumin is used. The competition of the markers and the drugs for two major high- and low-affinity binding sites is investigated. Using a mathematical procedure proposed by the same authors in a previous work the affinity constants of the binding drugs and markers for both types of site are calculated. An analogous behaviour is established for the three drugs—they have nearly the same affinity for the primary binding sites marked by phenylbutazon and diazepam and only one type of low-affinity site (diazepam-binding sites) is involved in binding. That can be explained assuming an overlapping sites.

Keywords: Indomethacin; Sulindak; Diclofenac sodium; Binding sites; Markers; Phenylbutazon; Diazepam; Affinity chromatography; Serum albumin

1. Introduction

Protein binding of drugs is involved in the general phenomenon concerning the interactions between small ligands and biopolymers. Such interactions are very often influenced by the presence of other small molecules—competitors. The ability of competitors to displace a ligand from the macromolecule can be expressed either by a simple competition or by more complex indirect mechanisms.

The binding of drugs to human serum albumin (HSA), the most important plasma protein, is a good model system to study interactions between small ligands and macromolecules. As the binding

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of a drug can be modified by the presence of other drugs, this is also a suitable model to study the competitive binding of two ligands to the albumin binding sites, where the displacement of one drug by another can take place. The investigation of drug-albumin binding phenomena helps to elucidate the drug–drug interactions in vivo.

The present work regards the binding and competitive behaviour of some drugs: indomethacin, sulindak and diclofenac sodium, which are all derivatives of arylacetic acids. They belong to NSAIDs—a therapeutic class that displays remarkable similarities in therapeutic actions and side effects, but is markedly heterogenous in structure.

Reports have been published on the binding of indomethacin to HSA and the nature of binding sites were discussed based on data obtained by means of ultrafiltration (Mason and McQueen, 1974) and circular dichroism (Ekman et al., 1980). The specificity of binding sites was studied with albumin immobilized in microparticles (Sjoholm et al., 1979; Sjoholm and Kober, 1980). A stoichiometric constant of binding was obtained by means of equilibrium dialysis and a stoichiometric analysis of data (Honore and Brodersen, 1984). Using difference spectroscopy an affinity constant was calculated and the binding model was proposed as well (Russeva and Mihailova, 1996). The variety of data are reported for indomethacin which in some cases are not in agreement. On the other hand only a single report exists about the albumin binding of sulindak. The affinity constants were determined for two classes of binding sites of sulindak in a circular dichroism study (Russeva et al., 1994). The nature of binding sites was investigated and a model of binding to the HSA molecule was proposed, too. The binding of C-labeled diclofenac-Na to human serum proteins was investigated in vitro by equilibrium dialysis and ultracentrifugation (Wagner and Sulc, 1979). The difference spectroscopy was used to study the influence of various factors on the albumin binding of diclofenac-Na and a model of binding was proposed as well (Russeva and Mihailova, 1996).

Previous studies have shown that the utilization of immobilized HSA in high performance liquid chromatography (HPLC) accurately simulates the binding properties of the free protein (Domenici et al., 1990, 1991). Data obtained have been shown to be precise and reproducible.

A high-performance liquid affinity chromatographic study based on chiral stationary phases (CSP) with immobilized (HSA) is reported in the present work to investigate the albumin binding behaviour of the drugs mentioned above. The location of binding sites was studied using diazepam (DAZ) and phenylbutazon (PBZ) as markers. The possibility to use these drugs as markers binding predominantly at each of the two major binding sites: site I and site II are described in our previous work (Russeva and Zhivkova, 1998). The results obtained indicate that two different binding sites exist for PBZ and DAZ on the HSA-CSP column: high- and low-affinity sites. A conclusion was derived from those data that the binding sites for PBZ and DAZ on the HSA-CSP column can not be differentiated entirely and that they are probably overlapping to some extent, so that common binding subsites are present.

2. Materials and methods

2.1. Drugs and chemicals

Indomethacin, sulindak, diclofenac-Na, phenylbutazon and diazepam are obtained from National Drugs Institute (Sofia, Bulgaria). Propan-1-ol for HPLC, as well as NaH_2PO_4 and Na_2HPO_4 of purest grade were provided from Merck (Darmstadt, Germany).

2.2. Chromatography

A modular HPLC system LC-10A Shimadzu (Japan) has been used, which consisted 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 controlled and the data were 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. Mathematical analysis

The retention factors k' at different marker concentrations [M] are used to calculate the quantitative parameters characterizing the binding process. A mathematical procedure concerning more than one type of binding sites is used (Zhivkova and Russeva, 1998). In this case the following equation is given:

$$k' = k'_{\mathrm{I}} + k'_{\mathrm{II}} + X = \frac{K^{\mathrm{I}}_{\mathrm{A}}[S^{\mathrm{I}}_{\mathrm{tot}}]}{1 + K^{\mathrm{I}}_{\mathrm{M}}[\mathrm{M}]} + \frac{K^{\mathrm{II}}_{\mathrm{A}}[S^{\mathrm{II}}_{\mathrm{tot}}]}{1 + K^{\mathrm{II}}_{\mathrm{M}}[\mathrm{M}]} + X. \quad (1)$$

k'_{I} and k'_{II} represent the parts of the retention factor k' due to binding to the primary (high-affinity) and secondary (low-affinity) binding site and $K^{\mathrm{I}}_{\mathrm{D}}$, $K^{\mathrm{II}}_{\mathrm{D}}$, $K^{\mathrm{I}}_{\mathrm{D}}$, $K^{\mathrm{II}}_{\mathrm{M}}$, $[S^{\mathrm{I}}_{\mathrm{tot}}]$ and $[S^{\mathrm{II}}_{\mathrm{tot}}]$ 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 marker does not bind.

3. Results and discussion

Although the investigated drugs belongs to one and the same therapeutic group they are different in their chemical structure and specificity. For that reason the considerations are given consequently for every individual ligand.

3.1. Indomethacin

As it can be seen from Fig. 1a at the very beginning the retention factor k' decreases linearly with the increasing of marker (PBZ) concentration, then (above 7.5 μM PBZ) it remains constant—the binding sites probably become saturated. The quantity of the displaced marker PBZ changes in the same manner during the experiment. The affinity constant determined for the binding of indomethacin to the sites where PBZ also seems to bind is high enough and corresponds to about 30% primary high-affinity binding (Table 1). The binding of indomethacin to sites marked by DAZ exhibits a biphase behaviour (Fig. 2a), what supports the hypotheses for binding to high- (about 9%) and low-affinity (about 85%) binding sites as well. There is considerable displacement of the marker (DAZ) at the higher concentration [M], too. The values of the high- and low-affinity constants (Table 1) exceed those of the marker (inhibitor) (Russeva and

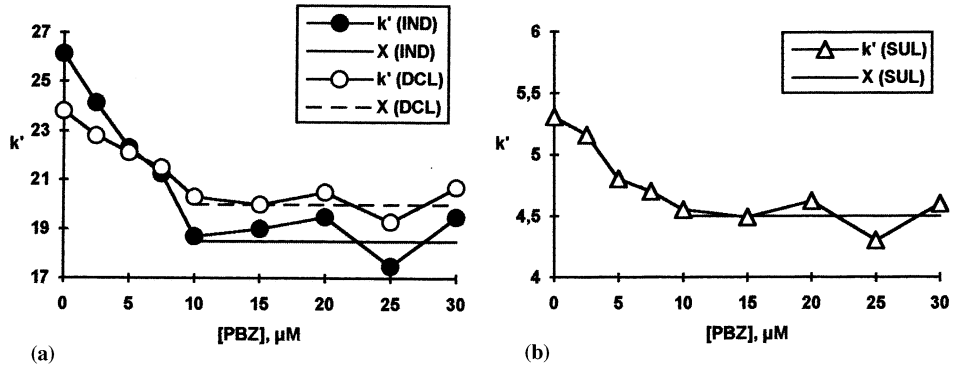

Fig. 1. Influence of the mobile phase concentration of the marker (PBZ) on the retention factors of the injected drugs: (a) indomethacin and diclofenac sodium; (b) sulindak. HPLC conditions: stationary phase—immobilized HSA-column 150 × 4.6 mm; eluent 67 mM phosphate buffer (pH 7.4)—propan-1-ol (92:8, v/v); column temperature 34 ± 0.1°C; flow-rate 1.2 ml/min; detection wavelengths 264 nm (for PBZ) and 320 (for the solvent).

Table 1
The affinity constants for the albumin binding of some NSAIDs to the sites marked by phenylbutazon (PBZ) and diazepam (DAZ)

Drug	Type of binding sites			
	High-affinity binding sites		Low-affinity binding sites	
	PBZ	DAZ	PBZ	DAZ
	K_A, M^{-1}			
Indomethacin	1.16×10^6	2.17×10^6	–	2.55×10^4
Sulindac	1.33×10^5	1.31×10^5	–	4.21×10^3
Diclofenac–Na	5.44×10^5	1.15×10^6	–	1.89×10^4

Zhivkova, 1998) for the same types of binding sites which illustrate the availability of the competitive effects.

3.2. Sulindak

Initially the retention factor k' decreases linearly until 10 μM concentration of the PBZ marker is reached, after which the values of k' remain constant (Fig. 1b). Binding to the primary (high-) affinity sites occurs as it can be seen from the value of the binding constant obtained (Table 1). In a different way sulindak binds to DAZ-binding sites: k' decreases continuously without saturation. The results (Table 1) support binding to the primary DAZ-binding sites (about 3%), to the secondary binding sites (about 80%) and to other sites (about 17%). A displacement of DAZ occurs in the whole range of marker concentration [M] due to the binding of sulindak to common sites. So sulindak binds to HSA in a manner similar to the binding of indomethacin.

3.3. Diclofenac sodium

The binding behaviour of diclofenac-Na is analogous to the binding of the drugs discussed till now. The experimental data confirm that when PBZ is used as a marker nearly 15% of the binding of diclofenac relates to the primary (high-affinity) PBZ binding sites. The retention factors remain constant above 7.5 μM marker concentration (Fig. 1b); in a similar way the quantity of the replaced PBZ initially arises until the mentioned concentration is reached and then remain unchanged. Diclofenac binds to the high- (nearly

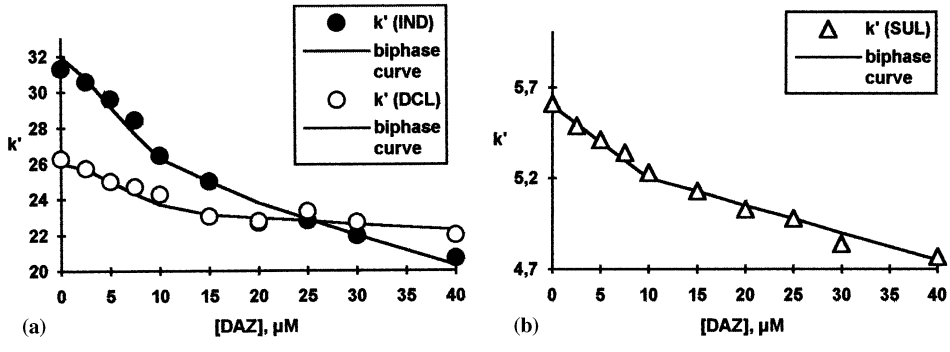

Fig. 2. Influence of the mobile phase concentration of the marker (DAZ) on the retention factors of the injected drugs: (a) indomethacin and diclofenac sodium; (b) sulindak. HPLC conditions: stationary phase—immobilized HSA-column 150 × 4.6 mm; eluent 67 mM phosphate buffer (pH 7.4)—propan-1-ol (92:8,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

Table 2
The affinity constants of the markers phenylbutazon (PBZ) and diazepam (DAZ) for high- and low-affinity binding sites of albumin in the presence of some NSAIDs

Drug	Type of binding sites			
	PBZ		DAZ	
	High-affinity $K_M \times 10^{-5}$ M^{-1}	Low-affinity	High-affinity $K_M \times 10^{-5}$ M^{-1}	Low-affinity $K_M.10^{-4}$ M^{-1}
Indomethacin	1.48 ± 0.63	–	0.72 ± 0.17	1.33 ± 0.18
Sulindac	1.84 ± 0.13	–	1.67 ± 0.26	0.55 ± 0.27
Diclofenac–Na	1.07 ± 0.85	–	0.90 ± 0.12	0.51 ± 0.12

5%) and low- (about 76%) affinity binding sites marked by DAZ, the affinity of the drug to both type of sites (Table 1) exceeds 10-fold the affinity of the marker itself—small quantities of DAZ are displaced. The affinity constants obtained for the binding of the marker DAZ are identical with those found for DAZ, used simultaneously as marker and analyte (Table 2). In the whole range of marker concentrations the retention factors decrease in a biphase manner.

4. Conclusions

The results concerning binding of indomethacin enable the conclusion that binding occurs to primary sites of both types with quite similar affinity constants. Secondary binding occurs only at the low affinity DAZ-binding sites. The affinity constants for the primary (high-affinity) binding of sulindak to the PBZ- and DAZ-binding sites are almost equal. There are also low-affinity sites, named DAZ-binding sites, where sulindak binds as well. Sulindak does not bind to the secondary (low-affinity) PBZ-sites. An analoguos behaviour was established regarding diclofenac-Na binding. In this case we find an affinity constant to high-affinity DAZ-binding sites exceeding twice the constant for the binding to the high-affinity PBZ-binding sites. The drug binds with a lower affinity to the secondary DAZ-sites. It was previously found that DAZ binds to the primary (high-affinity) binding sites of PBZ. In the cases when DAZ presents as a marker the low-affinity sites are shared with PBZ (Russeva and Zhivkova, 1998). Based on the hypothesis for a partial overlapping of sites it is easy to explain nearly the same affinity constants for the primary binding of the studied drugs to the sites marked by PBZ and DAZ. The binding of the three drugs only to one type of low-affinity sites (DAZ-sites) can well be explained assuming an entire overlapping of the secondary (low-affinity) PBZ- and DAZ-binding sites.

In a previous work (Russeva et al., 1994) the model of the stepwise binding was proposed to explain the interaction of sulindak with HSA studied by circular dichroism. The present results allow confirmation of this concept and to apply it for the other drugs using the HPLAC method as well. So, it can be concluded that the binding of the drugs under investigation goes stepwisely: initially with high affinity and then with a lower affinity occupying site with different nature according to the chemical structure and preferention of the individual ligands.

References

Domenici, E., Bertucci, C., Salvadori, P., Motellier, S., Wainer, I.W., 1990. Immobilized serum albumin: rapid HPLC probe of stereoselective protein binding interactions. Chirality 2, 263–268.

Domenici, E., Bertucci, C., Salvadori, P., Wainer, I.W., 1991. Use of a human serum albumin -based chiral stationary phase for high-performance liquid chromatography for the investigation of protein binding: detection of the allosteric interaction between warfarin and benzodiazepine binding sites. J. Pharm. Sci. 80, 164–169.

Ekman, B., Sjodin, T., Sjoholm, I., 1980. Binding of drugs to human serum albumin-XV. Characterization and identification of the binding sites of indomethacin. Biochem. Pharmacol. 29, 1759–1765.

Honore, B., Brodersen, R., 1984. Albumin binding of anti-inflammatory drugs. Utility of a site-oriented versus a stoichiometric analysis. Mol. Pharmacol. 25, 137–150.

Mason, R.W., McQueen, E.G., 1974. Protein binding of indomethacin: binding of indomethacin to human plasma albumin and its displacement from binding by ibuprofen, phenylbutazone and salicylate, in vitro. Pharmacology 12, 12–19.

Russeva, V., Stavreva, N., Rakovska, R., Michailova, D., 1994. Binding of sulindak to human serum albumin studied by circular dichroism. Arzneim.-Forsch./Drug Res. 44(I) (2), 159–162.

Russeva, V., Mihailova, D., 1996. Modelling of indometacin and diclofenac sodium binding to the molecule of human serum albumin. Arzneim.-Forsch./Drug Res. 46(I) (3), 288–292.

Russeva, V., Zhivkova, Z., 1998. Albumin binding sites studied by high-performance liquid affinity chromatography. Int. J. Pharm. 168, 23–29.

Sjoholm, I., Ekman, B., Kober, A., Ljungstedt-Pahlman, I., Seiving, B., Sjodin, T., 1979. Binding of drugs to human serum albumin: XI. The specificity of three binding sites as studied with albumin immobilized in microparticles. Mol. Pharmacol. 16, 767–777.

Sjoholm, I., Kober, A., 1980. Binding of drugs to human serum albumin with special reference to the binding of some anti-rheumatic agents. Br. J. Clin. Pract., Symp. Suppl. 6, 6–9.

Wagner, J., Sulc, M., 1979. Binding von Diclofenac-Na (Voltaren^{R}) an Serum-proteine verschiedener Spezies und Interaktionen mit anderen Pharmaka. Akt. Rheumatol. 4, 153–162.

Zhivkova, Z., Russeva, V., 1998. New mathematical approach for evaluating of drug binding to human serum albumin by high-performance liquid affinity chromatography. J. Chromatogr. B 707, 143–149.