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Journal of Chromatography B, 714 (1998) 277–283

JOURNAL OF CHROMATOGRAPHY B

Stereoselective binding of ketoprofen enantiomers to human serum albumin studied by high-performance liquid affinity chromatography

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Received 31 December 1997; received in revised form 17 April 1998; accepted 28 April 1998

Abstract

A chiral stationary phase based on immobilized human serum albumin (HSA) was used to study the stereoselective binding of ketoprofen enantiomers by means of high-performance liquid affinity chromatography. The technique of zonal elution was applied together with a novel mathematical approach describing attachment to more than one type of binding site. Phenylbutazon (PBZ) and diazepam (DAZ) were used as markers for the major believed binding regions on HSA. Both *R*- and *S*-ketoprofen (KT*R* and KT*S*) display high affinity to the primary PBZ- and DAZ-binding sites and low-affinity to the secondary DAZ sites. The binding to high-affinity regions is accepted to be a stepwise process initiated by the binding to the primary DAZ sites and followed by the attachment to the primary PBZ sites. The chiral recognition is attributed to the high-affinity PBZ-binding sites and to the low-affinity DAZ-binding sites.

Keywords: Enantiomer separation; Ketoprofen

1. Introduction

Ketoprofen is a nonsteroidal anti-inflammatory drug (NSAID) widely used clinically for treatment of arthritis and rheumatic diseases. It is applied as a racemate although its pharmacological activity similar to other arylpropionic acid derivatives is attributed to the *S*-enantiomer [1,2]. At normal therapeutic levels ketoprofen is more than 90% protein bound, predominantly with human serum albumin (HSA) [3]. Because of the clinical significance of such extensive protein binding different aspects of ketoprofen–HSA interactions have been a subject of research interest. The earlier studies agree that the drug binds primarily to site II (indole-benzodiazepine site) and secondarily to site I (warfarin–azapropazon–phenylbutazon site) on HSA [4–6]. These investigations used racemate mixture and did not mind the stereoselective aspects of drug–HSA interactions.

Actually the binding of racemic drugs to HSA is potentially stereoselective because of the inherent chirality of the protein. Enantioselectivity has been repeatedly suggested for warfarin [7,8], several NSAIDs [9–19], benzodiazepines [20,21], etc. It is believed that both major binding regions of HSA are able to distinguish between the optical antipodes [22]. The stereoselectivity may arise from different affinity of the enantiomers towards common sites as well as from preferences to different binding areas. The exact origin of the discrimination process is usually difficult to assess since conformational alter-

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0378-4347/98/$19.00
PII: S0378-4347(98)00211-4

ations caused by the optical antipodes are also possible [15,20,23]. To develop an accurate picture of racemate drug–protein interactions it is necessary to consider the binding behavior of the individual enantiomers.

Very few works concern the stereoselective aspects of ketoprofen binding to HSA. Studies with classical methods (equilibrium dialysis and ultrafiltration) established a lack [24] or slight difference [25,26] in the binding behavior of the enantiomers. On the contrary, recent investigations using high-performance liquid affinity chromatography (HPLAC) [15] and circular dichroism [27] suggest that in the presence of octanoic acid, HSA is able to distinguish between both optical antipodes [22,23]. Obviously further research is necessary to settle the contradictions and to clarify the enantioselective aspects of ketoprofen–HSA interactions.

The purpose of this work is to examine the binding of the individual enantiomers *R*- and *S*-ketoprofen to both major binding sites on HSA. Phenylbutazon and diazepam whose binding properties have been examined and discussed in detail in our previous study [28] are used as markers for site I and site II, respectively. The analytical method is HPLAC based on immobilized HSA chiral stationary phase (HSA–CSP). This approach was recently outlined as an optimal experimental strategy of the in vitro binding studies [29] and have been successfully used for studying various aspects of drug–HSA binding, including stereoselective phenomena [15–20,30–32].

2. Experimental

2.1. Reagents

Diazepam (DAZ), phenylbutazon (PBZ) and racemic ketoprofen (KT) were kindly supplied by National Drug Institute (Sofia, Bulgaria). *S*-ketoprofen (KT*S*) and *R*-Ketoprofen (KT*R*), 98.7% chemical purity and 99.8% enantiomeric excess were gifts from Menarini Pharmaceutical (Barcelona, Spain). Propan-1-ol for HPLC as well as the buffer components, NaH_2PO_4 and Na_2HPO_4 of purest grade, were purchased from Merck (Darmstadt, Germany). The HSA–CSP column (150×4.6 mm) was provided by Shandon Scientific (Runcorn, UK).

2.2. Chromatography

Chromatography was carried out isocratically on modular HPLC system LC-10A Shimadzu (Japan) arranged 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 analysis was controlled and the data were acquired with CLASS LC-10 software. Mobile phase composed of 67 m*M* potassium phosphate buffer and propan-1-ol (92:8 v/v) was pumped through the column at a flow-rate of 1.2 ml/min. The temperature was maintained at 34±0.1°C. Detection was performed at relevant λ_{max} for the unretained substance (injection solvent), DAZ, PBZ and KT. The technique of zonal elution was applied. Small quantity of the examined drug (analyte) was injected onto the HSA–CSP column while increasing concentration (0–30 μ*M*) of cobinding drug (marker) was added to the mobile phase. In the case of competition between marker and analyte for common binding sites the increase of marker concentration induces decrease in the retention of the analyte. The retention is expressed as capacity factor $k'=(t_R-t_0)/t_0$, where t_R and t_0 are the retention times of the analyte and the unretained compound. The enantioselectivity factors are calculated as a ratio of the capacity factors of both enantiomers.

2.3. Mathematical approach

The data are treated using the mathematical procedure proposed by Noctor et al. [15] and modified recently for the case of competition at more than one type of binding site [33]. The effect of increasing marker concentration was analyzed according to the equation

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

for single site competition, or

$$k' - X = k'_{I} + k'_{II} = \frac{K_A^{I}[S_{tot}]}{1 + K_M^{I}[M]} + \frac{K_A^{II}[S_{tot}]}{1 + K_M^{II}[M]} \quad (2)$$

for two types of binding site competition.

k' represents the capacity factor at different

marker concentrations [M], X is the part of the retention due to binding at sites accessible only for the analyte, k'_{I} and k'_{II} are the parts of the capacity factor k' due to binding at the primary (high-affinity) and the secondary (low-affinity) binding sites, and K^{I}_{A}, K^{II}_{A}, K^{I}_{M}, K^{II}_{M}, $[S_{tot}{}^{I}]$ and $[S_{tot}{}^{II}]$ are the respective values of the affinity constants for the analyte, marker and common binding sites concentrations. If the analyte and the marker compete for only one type of binding sites, the plot of $1/(k'-X)$ yields a straight line. K_M is obtained as a ratio of the slope to the intercept. If the concentration of common binding sites is known, K_A is also easy to calculate. In the case of two types of binding site competition the reciprocal of Eq. (2) represents a biphase curve. At lower marker concentration concomitant binding occurs for both high- and low-affinity binding sites. After the high-affinity sites (generally with lower concentration) have been occupied the competition continues exclusively for the low-affinity sites. For this final phase Eq. (2) is reduced to single site competition equation:

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

Using Eq. (3) the theoretical values of k'_{II} for the initial phase of HSA binding are calculated. The part of the retention due to binding to the high-affinity sites is determined by subtracting k'_{II} from k' and simple single site equation characterizing the high-affinity binding is also obtained:

$$k'_{I} \approx k' - X - k'_{II} = \frac{K^{I}_{A}[S^{I}_{tot}]}{1 + K^{I}_{M}[M]} \quad (4)$$

The affinity parameters for both types of binding are calculated using Eqs. (3) and (4) as described above. X-values are determined according the procedure given in our previous work [33].

3. Results

The binding behavior of KT and its enantiomers is examined in competitive studies using PBZ and DAZ as markers for site I and site II on an immobilized HSA–CSP chromatographic column. The capacity factors observed for enantiomers does not differ significantly when KT*S* or KT*R* are injected separately or as racemate. That is why all mathematical calculations are based on the data obtained for the individual antipodes.

The effect of increasing PBZ concentration on the chromatographic retention of KT*S* and KT*R* is shown in Fig. 1. The capacity factor decreases almost linearly in the concentration range 0–10 μ*M* followed by a relatively constant level. This value of k' is considered to be X since it is independent of marker concentration and thus corresponds to the part of the retention due to binding to sites inaccessible for PBZ. An additional peak appears in the chromatogram that corresponds to PBZ (proved by its retention time and UV-spectra). The amount of displaced PBZ decreases until the marker concentration of 7.5 μ*M* after which it remains constant.

The graph of $1/(k'-X)$ (with X values of 4 for KT*S* and 5.6 for KT*R*) versus PBZ concentration in the range 0–7.5 μ*M* is presented in Fig. 2. The experimental data for both enantiomers fit to linear plots described by Eq. (1). This behavior indicates competition between marker (PBZ) and analyte (KT*S* or KT*R*) for a single type of binding sites. The calculated marker affinity constant for the common binding sites are commensurate with that reported in our recent study for the primary PBZ-binding sites [28]. Most probably PBZ and KT enantiomers compete just for these high-affinity PBZ-binding sites. Using the previously established concentration of these sites [28], the affinity constants of KT*S* and KT*R* are determined according to Eq. (1). The binding parameters for marker [28] and for KT enantiomers are summarized in Table 1.

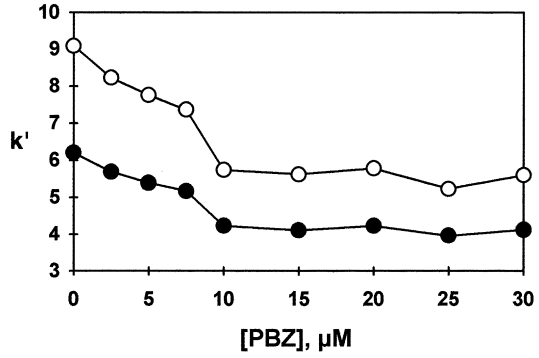

Fig. 1. Change in k' for KT*R* (○) and KT*S* (●) with mobile phase marker concentration. k' values remain almost constant at [PBZ] higher than 7.5 μ*M*.

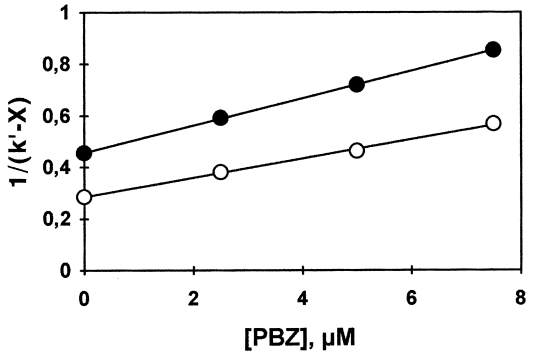

Fig. 2. Experimental $1/(k'-X)$ for KT*R* (○) and KTS (●) versus [PBZ] in the range 0–7.5 μ*M*, and theoretical plots. *X* values are 5.6 and 4 respectively. The equations of best-fit lines are $y=(0.285\pm0.005)+(37\,228.000\pm1143.210)x$ with $R=1.000$ for KT*R* and $y=(0.456\pm0.002)+(53\,028.000\pm345.091)x$ with $R=0.999$ for KT*S*.

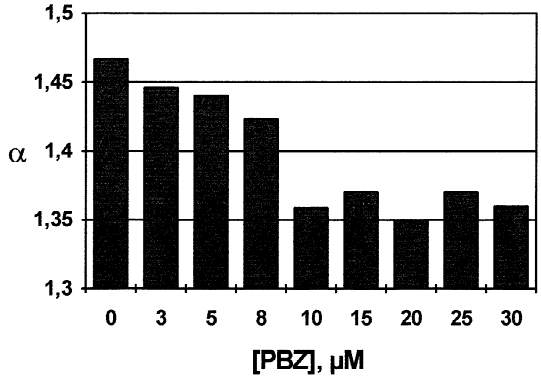

Fig. 3. Enantioselectivity factor as a function of marker concentration. α remains almost constant at [PBZ] higher than 7.5 μ*M*.

Fig. 3 presents the enantioselectivity factor α as a function of PBZ concentration. Like k', α decreases until a concentration of about 10 μ*M* is reached after which it remains almost constant.

Quite different behavior is observed during cobinding of both KT enantiomers with DAZ. k' values decreases in the whole concentration range in biphase manner without reaching saturation. Similar behavior was observed using DAZ both as marker and analyte [28], and two types of binding sites were defined with binding parameters listed in Table 2. Obviously KT enantiomers compete with DAZ for both types of DAZ-binding sites. The *X* values of 0.44 and 0.61 are calculated as described previously [33] plotting $1/k'$ against DAZ for the final part of the curve and assuming that marker concentration ensuring binding sites saturation is equal to the concentration of the low-affinity DAZ-binding sites. The corresponding graphs of $1/k'-X)$ versus DAZ are presented in Fig. 4. The mathematical analysis of the relationship is performed as described above. The following parameters are determined consequently:

- the affinity constants of marker and analyte for the low-affinity sites of competition using the reciprocal of Eq. (3) and the concentration of these sites according [28];
- the theoretical values of k'_{II} and k'_{I} for the initial phase of the binding process;
- the affinity constants of marker and analyte for the high-affinity sites according the reciprocal of

Table 1
Binding characteristics of ketoprofen enantiomers for PBZ-binding sites. Parameters for PBZ obtained in previous study [28] are presented

Analyte	Binding sites concentration (*M*)	Marker affinity constant (M^{-1})	Analytes affinity constant (*M*)	Part of the retention due to binding at this sites (%)
PBZ	$6.40\cdot10^{-6}$ (±0.22)	$1.84\cdot10^{5}$ (±0.13)	$1.84\cdot10^{5}$ (±0.13)	15
KTS	–	$1.16\cdot10^{5}$ (±0.02)	$3.42\cdot10^{5}$ (±0.13)	35.5
KT*R*	–	$1.31\cdot10^{5}$ (±0.06)	$5.50\cdot10^{5}$ (±0.17)	38

Table 2
Binding characteristics of ketoprofen enantiomers for DAZ-binding sites. Parameters for DAZ obtained in previous study [28] are presented

Type of binding sites	Analyte	Binding sites concentration (M)	Marker affinity constant (M^{-1})	Analytes affinity constant	Part of the retention,due to binding at this site (%)
High affinity	DAZ	$1.20\cdot10^{-6}$ (±0.15)	$0.97\cdot10^{5}$ (±0.18)	$0.97\cdot10^{5}$ (±0.18)	3
	KT*S*	–	$1.47\cdot10^{5}$ (±0.79)	$1.02\cdot10^{6}$ (±0.15)	14
	KT*R*	–	$0.75\cdot10^{5}$ (±0.33)	$1.11\cdot10^{6}$ (±0.12)	10
Low affinity	DAZ	$1.06\cdot10^{-3}$ (±0.15)	$4.24\cdot10^{3}$ (±0.58)	$4.24\cdot10^{3}$ (±0.58)	97
	KT*S*	–	$1.62\cdot10^{4}$ (±0.12)	$6.51\cdot10^{3}$ (±1.25)	81
	KT*R*	–	$1.97\cdot10^{4}$ (±0.20)	$9.63\cdot10^{3}$ (±1.31)	85

Eq. (4) and the concentration of these sites already known [28].

The binding parameters of KT enantiomers are summarized in Table 2.

Displacement of DAZ (identified by the UV spectra of the chromatographic peak and retention) is also observed when both enantiomers are injected onto the column, and the displaced amount remains almost constant in the whole range of DAZ concentrations. Similar independence from marker concentration has been observed for the enantioselectivity factor α.

4. Discussion

The results presented in this work indicate that both ketoprofen enantiomers bind to PBZ- as well as to DAZ-binding sites. Since it is known that PBZ engaged predominantly site I of HSA, and DAZ is a prototype for site II our data are in good agreement

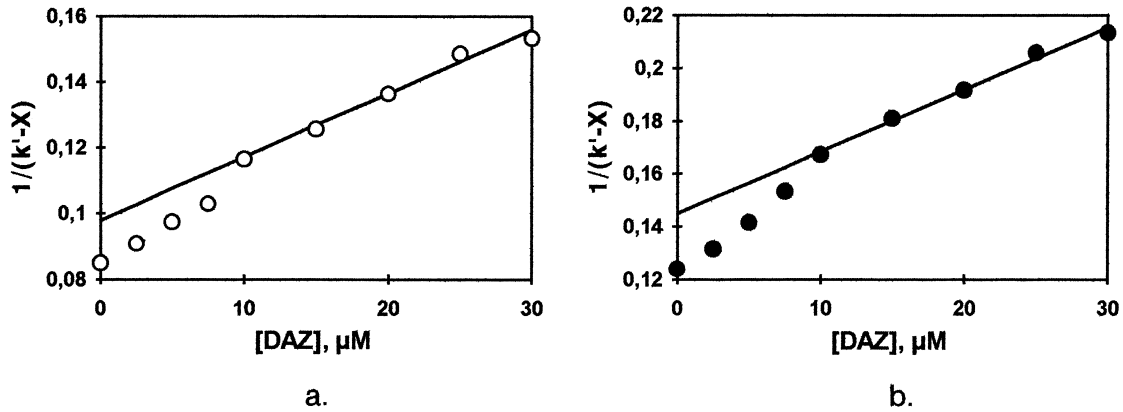

Fig. 4. Change in $1/(k'-X)$ of KT enantiomers with mobile phase marker (DAZ) concentration. (a) *R*-ketoprofen: (○) experimental data. Bilinear behavior is obvious. (—) theoretical plot for the final phase of the curve ([DAZ] in the range of 10–30 μM). The best fit-line is $y=(0.098\pm0.030)+(1930.000\pm130.675)x$ with a correlation coefficient of 0.993 ($n=5$). The X value is 0.61. (b) *S*-Ketoprofen: (●) experimental data. Bilinear behavior is obvious. (—) theoretical plot for the final phase of the curve ([DAZ] in the range of 10–30 μM). The best fit-line is $y=(0.145\pm0.003)+(2346.000\pm122.436)x$ with a correlation coefficient of 0.996 ($n=5$). The X value is 0.44.

with those previously reported [4–6]. KT*S* (as well as KT*R*) compete with PBZ only for the high-affinity PBZ-binding sites. This is supported by several observations:

- saturation of binding sites is achieved at marker concentration higher than 7.5 μM that coincides the previously reported primary PBZ-binding sites concentration (Table 1)
- the maximal level of displaced PBZ corresponds to the same marker concentration
- calculated values of PBZ affinity constants are in the same order with those obtained when PBZ was used both as marker and analyte (Table 1). Competitive effects may be responsible for the small observed differences.

The part of the retention of both enantiomers due to binding at the high-affinity PBZ sites is almost equal but the affinity constants although in the same order differ significantly. Moreover, Fig. 3 shows that the increase of marker concentration affects the binding of KT*R* to PBZ sites more strongly. On the basis of these findings it can be concluded that the high-affinity binding sites of PBZ are stereoselective towards KT enantiomers, KT*R* being preferred. This corresponds with earlier results [25,26] which report that KT*R* binds more strongly to HSA than KT*S*. Different binding behavior of the KT forms was also found by Noctor et al. [15] in their competitive study using as a marker the typical site I drug octanoic acid. They also observed a biphase relationship between marker concentration and chromatographic retention. The enantioselectivity factor increased significantly at lower marker concentration followed by a loss of chiral recognition. Although the mechanism of competition with octanoic acid is quite different the enantioselectivity of site I is obvious.

Both KT enantiomers compete with DAZ for the two proposed types of DAZ-binding sites since the increase in marker concentration results in a biphase decline of the retention of the analyte. The relatively high concentration of the secondary DAZ sites (Table 2) prevents the saturation of binding sites. The marker affinity constants for the primary DAZ sites are identical with those obtained when DAZ is marker and analyte concomitantly. However, in the presence of KT*S* or KT*R* the affinity constant of DAZ for the low-affinity binding sites increases visible, probably due conformational changes provoked by the analyte.

The high affinity DAZ-binding sites seems not to be enantioselective. Both KT antipodes display equal affinity towards them, much higher as compared with DAZ. It could be concluded that the displacement of DAZ occurs from these sites. On the contrary, the binding of KT*S* and KT*R* to the low affinity DAZ sites appears to be enantioselective, KT*R* being preferred. The affinity constants of the enantiomers are however 4–5 times lower than that of DAZ; that is why neither KT*S* nor KT*R* displace DAZ from these widely represented binding sites.

The attachment to DAZ sites is responsible for approximately 95% of the chromatographic retention for both enantiomers and very small differences are observed in the preference for a certain type of binding site.

The present results allow us to propose that both KT enantiomers display a high affinity to the primary PBZ- and DAZ-binding areas on HSA and bind with a low affinity to the secondary DAZ-binding regions. The attachment of KT*S* and KT*R* at the two types of primary binding areas may occur in a stepwise manner — initially binding to DAZ sites followed by association with PBZ sites. This hypothesis is in good agreement with the previously reported affinity constants of $0.37 \cdot 10^{7}\ M^{-1}$ and $0.5 \cdot 10^{5}\ M^{-1}$, respectively for two types of binding sites, found by means of circular dichroism [6]. The binding to the primary PBZ site and to the secondary DAZ sites is responsible for the chiral recognition. Actually, these binding areas partially overlap as has been discussed in our previous paper [28].

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