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DIFFERENT BINDING OF PROPRANOLOL ENANTIOMERS TO HUMAN ALPHA$_1$-ACID GLYCOPROTEIN

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Abstract—The binding of propranolol enantiomers to human alpha$_1$-acid glycoprotein was studied using high performance liquid chromatography in order to provide insight into binding models and to describe individual binding parameters of both enantiomers. The binding of (−)-propranolol was shown to be saturable with one major binding site ($n = 0.81$, $k = 2.73 \times 10^5$/M). The saturation process achieved its upper asymptotic value at drug/protein molar ratio of approximately 1. In the case of the opposite (+)-enantiomer the binding isotherm did not show evidence of saturation even at higher drug/protein molar ratios (up to 50). The individual binding parameters for (+)-enantiomer were $n = 0.38$, $k = 3.4 \times 10^6$/M and $n'k' = 1.39 \times 10^4$/M for the saturable and nonsaturable binding component, respectively. At drug/protein molar ratio 2 the circular dichroism measurements confirmed the existence of different binding models for individual propranolol enantiomers.

Observations on stereoselective interactions of some drugs with plasma protein binding sites have provided an important aspect in characterizing the specificity, selectivity and saturability of these binding sites (for review see [1, 2]). Although differences between drug enantiomers in affinity for plasma proteins are not so great as the differences between enantiomers in their affinities for a given pharmacological receptor, these differences in protein binding can be important particularly in pathophysiologically determined variability in the expression of individual proteins.

In contrast to the much better investigated human serum albumin, only a few reports have been published on stereoselective binding of drugs to human alpha$_1$-acid glycoprotein (AAG) recognized as the major plasma binding protein for basic drugs. Recent reports [3–7] showed that the fitting of optical isomers of some basic drugs to human AAG was unequal and manifested in differential binding strength and different distribution between bound and free states. For propranolol greater binding of (−)-enantiomer was demonstrated both to human plasma and to isolated human AAG [3, 4, 7].

The aim of the present study was to determine the binding models for (−)- and (+)-enantiomer by their interaction with human AAG and to describe binding parameters for individual enantiomers of propranolol. Binding experiments using both nonlabeled (−)- and (+)-propranolol were measured by high performance liquid chromatography and the resulting binding data were evaluated according to the affinity spectra method. Additionally circular dichroism measurements were performed that may give a useful indication of the nature and dynamics of the studied binding sites.

MATERIALS AND METHODS

Material and chemicals. Human alpha$_1$-acid glycoprotein was purchased from Behring Institute (F.R.G., purity 99%) and was used without further purification. (−)- and (+)-propranolol were kindly supplied by ICI (Macclesfield, U.K.). KH_2PO_4 and $Na_2HPO_4 . 12\ H_2O$ were of analytical grade. Water used for phosphate buffer was of Nanopure quality.

High performance liquid chromatography (HPLC). The HPLC experiments were performed with high pressure pump (HPP 4001, Laboratorní Přístroje, Prague, Czechoslovakia), an eight-port switching valve equipped with 25 and 100 μl loops (Model PK 1, Vývojové dílny, Czechoslovak Academy of Sciences, Prague, Czechoslovakia), a "compact glass cartridge" column (3.3 mm i.d. × 15 cm) packed with LiChrosorb Diol (Merck, Darmstadt, F.R.G., mean particle size 5 μm) and a variable-wavelength detector (Spectra-Physics, Model 770; LC Spectrophotometer Waters Lambda-Max Model 481; U.S.A.). Binding experiments were carried out at 37°. The mobile phases were formed from water solutions of 0.067 M KH_2PO_4 and $Na_2HPO_4 \cdot 12H_2O$ (pH 7.4) with addition of propranolol in the concentration range 2.5×10^{-7}–5×10^{-4} M for (−)-enantiomer and 5×10^{-7}–5×10^{-4} M for (+)-propranolol. The flow rate of the drug eluents, "degassed" by purging them with helium, was constant at 0.5 ml/min (for higher drug concentrations $\geq 1 \times 10^{-5}$ M) and at 0.9 ml/min (for lower drug concentrations $< 1 \times 10^{-5}$ M). For dilute solutions detection was at 212 nm, i.e. the maximum peak absorption of propranolol, or at 292 nm, when propranolol concentrations were high ($\geq 1 \times 10^{-4}$ M). The samples injected were solutions of human AAG (10 μM) in phosphate buffer containing various amounts of (−)- or (+)-propranolol. The binding of (−)- and (+)-propranolol to AAG was measured by using a drug solution saturating a nonchiral high-performance size exclusion column according to the method of Hummel and Dreyer [8]: injection of a protein sample into the eluent onto the column led to a positive peak corresponding to the

drug–protein complex and a negative "drug" peak representing the drug removed from the eluent by binding to the protein (method fully described in [8, 9]). In order to quantify the drug binding, an internal calibration process was used. By plotting the height of the "drug" peak as a function of the concentration of propranolol in the sample and interpolating it to zero, the concentration of the bound drug was observed, representing the exact excess of the drug injected relative to the actual eluent concentration (examples and figures also in [8, 9]).

Circular dichroism (CD). CD spectra were obtained using a DICHROGRAPHE III (JOBIN-YVON, France) calibrated with (+)-camphorsulfonic acid. Measurements were made in 5 mm cells using a 0.067 M phosphate buffer of pH 7.4. The alpha$_1$-acid glycoprotein concentration of 20 μM was used throughout. The solutions examined were prepared by saturating the AAG solution with drug up to drug/protein molar ratio [D/P] = 2. Results are expressed as molar ellipticity of difference spectra ($\Delta[\theta]$ = deg cm^2 dmol^{-1}) calculated with reference to the AAG concentration using the molecular weight of 44,100 Da. All induced CD curves became negative at lower wavelengths, however the high dynode voltage and the increasing intrinsic ellipticity of the AAG molecule did not permit detailed investigations at these wavelengths.

Evaluation of binding data. The binding data were evaluated by Scatchard analysis. The iterative program NONLIN [10] was used for the weighted nonlinear least-squares curve fitting. The best fit was obtained using statistical weight equal to $1/B_i^2$. We adapted the affinity spectra method proposed by Tobler and Engel [11]. For equilibrium binding isotherms the affinity spectra are defined as a plot of the number of binding sites against their corresponding dissociation constants. The parameters to be estimated, i.e. the number of binding sites and the dissociation constants, are obtained as the results of calculations that are based on the principle of least-square errors.

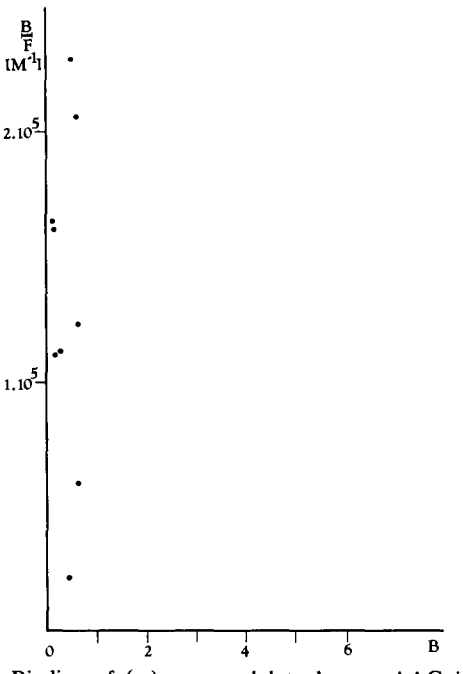

Fig. 1. Binding of (−)-propranolol to human AAG in Scatchard interpretation.

RESULTS

HPLC

Figures 1 and 2 show the experimental points obtained for (−)-propranolol in Scatchard and Klotz (i.e. B vs log F, [12]) interpretation respectively. Inspection of these experimental data revealed that (−)-enantiomer is bound to the AAG to one major site in a saturable manner. The saturation of AAG in the presence of (−)-propranolol achieved its upper asymptotic value at drug/protein molar ratio [D/P] = 1. At higher free drug concentrations (2.5×10^{-5}–5×10^{-4} M, i.e. [D/P] = 2.5–50) the values of the bound drug could not be determined under the described experimental conditions. The n (number of binding sites bound by a mole of protein) and k (affinity constant) characterizing the binding of (−)-propranolol were 0.81 and 2.73×10^5/M, respectively. The calculations were performed for [D/P] up to 1. Figures 3 and 4 analogously show the experimental points for (+)-propranolol–AAG interaction measured practically in the same concentration range as for (−)-propranolol. Scatchard (Fig. 3) and Klotz (Fig. 4) plots indicated the presence of nonspecific binding additionally to the primary binding site. This nonspecific binding is represented probably by low affinity, high capacity binding site(s). Up to the terminal experimental concentration (5×10^{-4} M) there was no evidence of saturation of these secondary binding sites. The n and k characterizing the (+)-propranolol binding to AAG are 0.38 and 3.39×10^6/M, respectively, the nonspecific binding is characterized by $n'k' = 1.39 \times 10^4$/M.

The results described above clearly show different binding models for (−)- and (+)-propranolol interaction with human AAG. On the model basis proposed by Tobler and Engel [11] the binding isotherm for (−)-propranolol is described by the equation

$$B = \sum_{i=1}^{z} \frac{n_i k_i F}{1 + k_i F} \text{ with } z = 1 \qquad (1)$$

and the binding isotherm for (+)-propranolol with the equation

$$B = \sum_{j=1}^{z} \frac{n_j k_j F}{1 + k_j F} + n'k'F \qquad (2)$$

where B is the concentration of drug bound by a mole of the protein and F is the concentration of the free drug. The term $n'k'F$ represents the nonspecific binding at secondary low affinity high capacity binding sites.

CD

The interaction between human AAG and propranolol enantiomers was followed qualitatively by CD on the basis of AAG spectra in the presence of

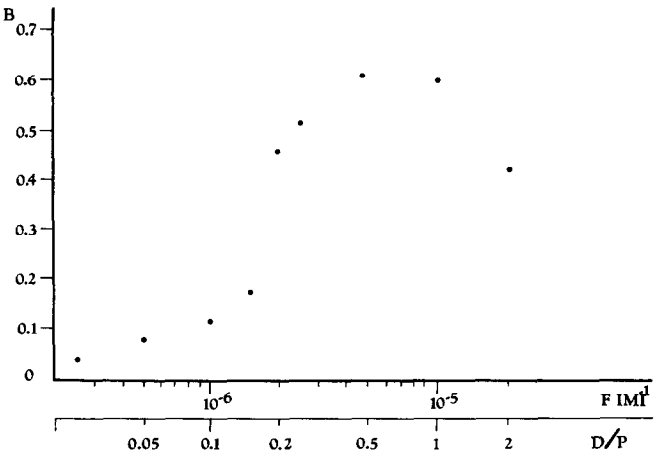

Fig. 2. Binding of (−)-propranolol to human AAG in Klotz [12] interpretation ([D/P], drug/protein molar ratio).

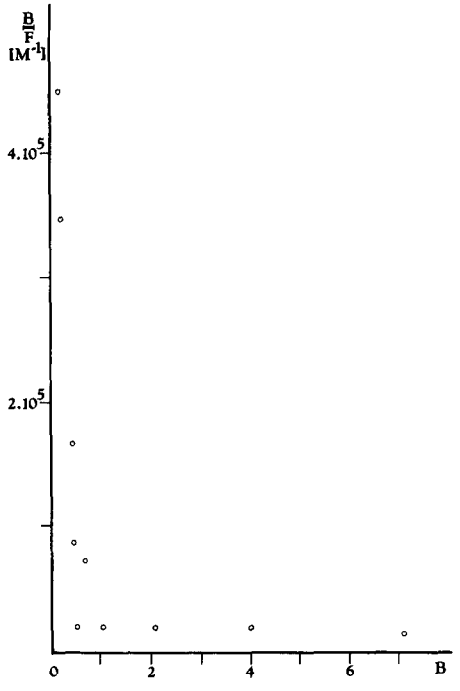

Fig. 3. Binding of (+)-propranolol to human AAG in Scatchard interpretation.

drug molecules in the wavelength regions of 350–250 nm and of 210–250 nm. The changes produced can be better seen on the difference spectra presented in Fig. 5. Here the AAG spectrum was subtracted from the spectrum obtained with AAG in the presence of 40 μM ([D/P] = 2) of (−)- and (+)-propranolol respectively: CD spectra of the AAG molecule alone showed two positive peaks at 290 and 260 nm. In the presence of (−)-enantiomer we could detect also two positive peaks at 295 and 260 nm on the differential spectrum. However the CD spectrum of the AAG solution in the presence of (+)-propranolol was composed of new negative bands with maxima at 325, 305 and 280 nm. These new extrinsic Cotton effects due to dichroic absorption at 325, 305 and 280 nm provided evidence for a more pronounced molecular interaction between AAG and (+)-propranolol than in the case of (−)-enantiomer. The interaction of AAG and (+)-propranolol was followed by conformational change of the protein molecule. The bands at 325 and 305 nm can be ascribed to the extrinsic Cotton effect due to binding of (+)-propranolol to the protein surface (i.e. non-specific partition-like binding). The changes produced in the wavelength region 210–250 nm were not significant. Also at lower drug/protein molar ratios ([D/P] < 2) there was no evidence of changes in the differential spectra of individual propranolol enantiomers.

DISCUSSION

Several investigators have studied the binding of racemic propranolol to the AAG molecule using different experimental methods. Some of them proposed the model composed of one class of binding sites [13, 14], others [15–18] reported binding to two classes of binding sites. The latter group concluded that the binding to the second binding site did not show evidence of saturation. Previous experiments with racemic mixture confirmed the concept of the presence of a nonsaturable binding component [16]. The pharmacokinetically relevant question whether the binding of propranolol enantiomers to chirally constituted AAG exhibits some degree of stereoselectivity has only recently been considered. Walle *et al.* [3] were first to report in human plasma and also with isolated proteins stereoselective binding of the pharmacologically more potent (−)-enantiomer using equilibrium dialysis. The results of Albani *et al.* [4] applying a combined equilibrium dialysis–liquid chromatographic technique and of Brunner and Müller [7] using the indirect method common in receptor–radioligand binding studies are in agreement with the previous report [3]. In the above-mentioned studies with isolated AAG [3, 4, 7]

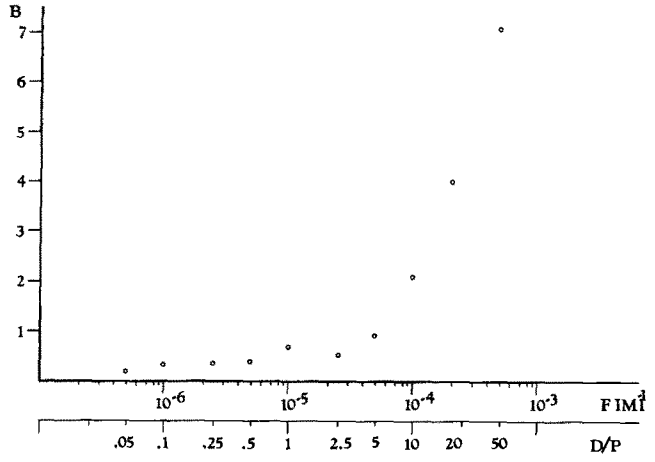

Fig. 4. Binding of (+)-propranolol to human AAG in Klotz [12] interpretation ([D/P], drug/protein molar ratio).

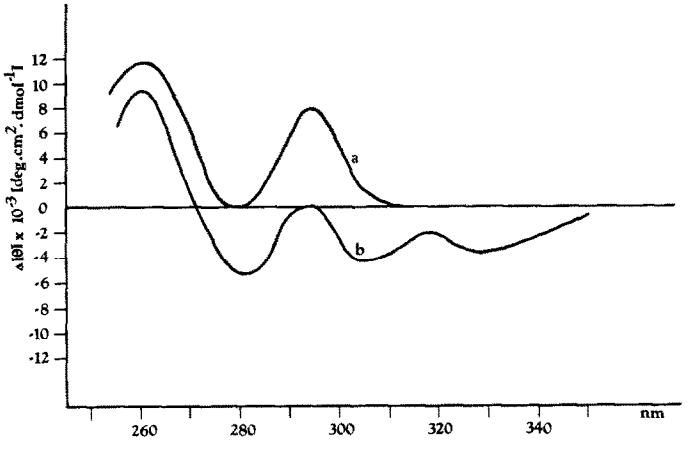

Fig. 5. Differential CD spectra of (−)propranolol (a) and (+)-propranolol (b) interaction with human AAG (20 μM) at drug/protein molar ratio 2.

only the values of the unbound fraction (22 ± 2% [3] and 23.0 ± 1% [4], and of the stereoselective ratio for the unbound fraction f_u (+)/(−) propranolol (1.27 [3], 1.31 [4] as well as 1.3 and 1.4 [7]) were determined.

In our present study using the Hummel–Dreyer method and high performance liquid chromatography we detected that the binding of (−)-propranolol to human AAG was evidently different from the binding observed in the case of the opposite (+)-enantiomer. The binding model observed for (+)-propranolol shows some similarities to the binding model of racemic mixture of both enantiomers. However, comparison of individual binding constants revealed discrepancies in the values of n and k between (+)- and (±)-propranolol. Although the binding parameters for (−)-enantiomer ($k = 2.73 \times 10^5$/M, $n = 0.81$) are within the range of values reported for racemic propranolol [13–18], the (+)-enantiomer binding parameters indicated the presence of a binding site of higher affinity and lower capacity ($k = 3.4 \times 10^6$/M, $n = 0.38$) than mentioned in the literature [15–18]. Subsequent experimental studies confirmed the data presented in Figs 1–4. The substantial difference in binding models between enantiomers of propranolol resides in the saturability of the primary binding site, i.e. in the presence of nonspecific binding in the case of (+)-propranolol (characterized by $n'k' = 1.39 \times 10^4$/M). The binding site for (−)-propranolol clearly showed saturation phenomena (possibly due to internalization of the ligand) at drug/protein molar ratio approximately 1. It can be concluded that the (+)-enantiomer is exclusively responsible for the nonsaturable binding component in racemic propranolol.

In the light of our present results there is a common binding site for both enantiomers on the AAG molecule. The essential difference in the binding models consists probably in different spatial arrangement of the protein molecule in the presence of individual enantiomers. Different conformational states of the

protein molecule resulting from different kinds of AAG interaction with propranolol enantiomers were supported in our study by using circular dichroic spectra. The results presented in Fig. 5 show that the flexible protein structure assumes different conformations depending upon the nature of the enantiomer present. Our results from CD spectra speak in favor of the concept of different modes of interaction between human AAG and propranolol enantiomers. The changes detected in (+)-propranolol confirmed in addition to conformational changes the presence of a nonspecific binding component (CD spectra 350–300 nm) appearing solely in this enantiomer.

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