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Propafenone binding interaction with human α_1-acid glycoprotein: assessing experimental design and data evaluation

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Abstract: Binding data on racemic RS-propafenone as well as individual R- and S-drug enantiomers interacting reversibly with human α_1-acid glycoprotein, as obtained by a high-performance liquid chromatographic method, are evaluated according to three different approaches introduced, respectively, by Scatchard, Bjerrum, and by Tobler and Engel. A non-linear curve-fitting procedure was applied to compute the binding parameters exclusively for the binary system comprising the examined protein and R- and S-propafenone, individually. The exactness of the study design rather than the numerical values were the focus of attention in the evaluation of the data found.

Keywords: *RS-propafenone; propafenone enantiomers; α_1-acid glycoprotein; drug-protein interaction; binding data evaluation.*

Introduction

The enantiomers of a racemic drug could, and often do, have different biological activities as a consequence of their selective, stereochemical interaction with macrobiomolecules in the living organisms [1]. This is why chemists and pharmacologists have become increasingly concerned with enantiomer–protein interactions [2, 3]. Enantiospecificity in pharmacokinetics arises because of enantioselectivity in one or more of the processes involved, i.e. drug absorption, distribution, metabolism, and excretion [4]. For R- and S-propafenone enantiomers, for instance, different pharmacological activities [5] and pharmacokinetic parameters as well [6] have been observed.

The extent of binding of the antiarrhythmic drug propafenone to human plasma proteins is known to be high, with α_1-acid glycoprotein (α_1-AGP) playing a major role in this reversible binding interaction [7]. Using a simple "Scatchard plot" graphical analysis for RS-propafenone, Gillis *et al.* [8] postulated two different classes of specific, saturable, binding sites on human α_1-AGP.

The present paper reports on the application of a high-performance liquid chromatographic (HPLC) method [9, 10] for studying the reversible interacting system comprising RS-, R-, or S-propafenone and the human α_1-acid glycoprotein. Along with assessing different modes of binding data evaluation, attention was focused also on the exactness of the applied experimental design itself.

Materials and Methods

Chemicals

Human α_1-acid glycoprotein (G 9885; mol. wt. considered to be 44.1 kDa) was purchased from the Sigma Chemical Co. (St Louis, MO, USA). RS-propafenone hydrochloride as well as both its R- and S- enantiomers (as HCl salts), with an optical purity of 98.4 and 99.0%, respectively, were kindly supplied by Dr W. Lindner (Karl-Franzens-University, Graz, Austria). The buffer components KH_2PO_4 and $Na_2HPO_4 \cdot 12\ H_2O$ were of p.a. purity grade (Merck, Darmstadt, Germany). The water was of Millipore Q quality (Millipore Corporation, Bedford, MA, USA).

Chromatography

The HPLC experiments were performed by an instrument consisting of a high-pressure

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syringe pump (HPP 5001; Laboratorní přístroje, Prague, Czech Republic), an eight-port switching valve equipped with 25- and 100-μl loops (PK 1; Vývojové dílny, Czech Academy of Sciences, Prague), a "compact glass cartridge" column (CGC; 15 cm × 3.3 mm) packed with LiChrosorb Diol (Merck, Darmstadt; mean particle size 5 μm), and a variable wavelength photometric detector (Lambda-Max 481; Waters Assoc., Inc., Milford, MA, USA). All experiments were carried out at 37.0°C. The mobile phases applied were phosphate buffered (0.067 mol l^{-1}, pH 7.4) solutions of RS-propafenone in the concentration range of 2.5×10^{-7}–5.0×10^{-4} mol l^{-1} or in that of 5.0×10^{-7}–5.0×10^{-4} mol l^{-1} for the R- and S-propafenone enantiomers, respectively. For the diluted drug solutions ($<1.0 \times 10^{-4}$ mol l^{-1}) the detection was set at 251 nm, whereas at the drug concentrations $\geq 1.0 \times 10^{-4}$ mol l^{-1} the setting was at 274 nm. The flow rates of the eluents, which were degassed by helium, were in the range 0.5–1.3 ml min^{-1}. The samples injected were solutions of human α_1-AGP (10.0 μmol l^{-1}; i.e. 441 μg ml^{-1}) in phosphate buffer (0.067 mol l^{-1}, pH 7.4) containing various amounts of RS-, R-, or S-propafenone.

The injection of such a sample yields two peaks. The first (positive) peak belongs to the protein–drug complex. Its retention volume equals the void volume of the chromatographic column used. The second, negatively or positively oriented peak, which is detected at the retention volume of the drug, manifests the ligand deficit or excess in the sample analysed. The purpose of using this so called Hummel and Dreyer internal calibration technique [9, 10] is to find such a concentration of the ligand which just compensates the drug deficit in the sample analysed or to determine that ligand excess which eliminates the appearance of the second peak on the chromatographic record.

Binding data evaluation

Scatchard plot. The binding isotherm of a ligand interacting exclusively with a single class of specific, saturable, binding sites on the macrobiomolecule/protein is usually described by the following equation:

$$B = nkF/(1 + kF), \qquad (1)$$

where B is the number of moles of the ligand bound per one mole of the protein, n represents the total number of binding sites, k characterizes the association constant of the given ligand against particular binding sites on the protein, and F is the molar concentration of the free, unbound ligand fraction.

Equation (1) or its manipulated form $B/F = nk - kB$ [11] is frequently used for the graphical evaluation of the drug binding to the receptor. The linear dependence B/F vs B, simply called also "Scatchard plot", has the following characteristics [12]:

— the negatively taken direction of the line is equal to the value of the association constant k;

— the intercept on the abscissa equals the value of the total number of binding sites on the protein, n.

Bjerrum plot. The presentation of the binding data in the form of the so-called "Bjerrum plot", B vs log$F$ [13], proved to be especially advantageous for the assessment of the saturability of the examined binding interaction [12, 14]. The B vs log$F$ dependence has also some characteristic features [12]:

— the S-shaped functional dependence is symmetric with respect to its middle, i.e. to its inflection point;

— the localization of the inflection point in relation to the ordinate (B) represents exactly one half of the total number of binding sites on the protein, i.e. $n_{1/2}$;

— on saturating all binding sites, that is when the concentration of the free ligand fraction is approaching an infinitely high level, the ordinate value (B) of the S-shaped curve reaches the value equal to n.

Tobler and Engel plot. In 1983 Tobler and Engel published an original procedure of computer analysis of equilibrium binding experiments the result of which is the so-called Affinity spectrum [15]. This spectrum (plot) shows the number of binding sites vs the corresponding dissociation constants ($1/k_i$).

The input data are represented exclusively by the set of values F_i and B_i. Their computer treatment demands neither iteration starting parameters nor a mathematical model to be supplied, and is derived from a general binding isotherm description:

$$B = \sum_{i=1}^{N} n_i k_i F/(1 + k_i F) + n'k'F + k_0, \qquad (2)$$

where B represents the number of moles of the

drug bound by one mole of the protein, F is the free drug molar concentration, k_i characterizes the succession of association constant values with sufficient density throughout the interval of interest (in our case $N = 100$; $F = 2.5 \times 10^{-7}$ or 5.0×10^{-7} up to 5.0×10^{-4} mol l^{-1} for the racemate or propafenone enantiomers), and n_i, the only variable treated as unknown, is the number of binding sites. The terms $n'k'$ and k_0 represent the non-specific and irreversible interaction components, respectively.

The result of computation, based on the linear programming principle, is that n_i is significantly greater than zero for only some k_i values. Statistical evaluation produces one (or more) bell-shaped curve(s) the features of which provide information concerning the most probable model of the interaction, i.e. one (or more) class(es) of specific, saturable, binding sites and an indication if non-specific, unsaturable, as well as irreversible binding components have come into play and should thus be accounted for. The location and the width of the bell-shaped curve correspond to the affinity (in the term of the dissociation constant $1/k_i$) of the given class of the specific binding sites represented by k_i and to the error of this value, respectively, while the curve height relates to the value of the number of binding sites, n_i [15].

Results

Figure 1, upper panel, represents the reversible binding interaction between RS-propafenone and the human α_1-AGP, plotted as discrete points in the Scatchard presentation (B/F vs B). The lower panel of Fig. 1 shows the binding data observed for both R- and S-drug enantiomers interacting with the same protein. From the evidently curvilinear shape of each isotherm one may exclude the possibility of classifying the binding interaction between propafenone (either that of the racemate or a single enantiomer) and the human α_1-AGP as specific, saturable, involving exclusively one single class of binding sites on the protein.

Such a statement is also unequivocally supported by the trends of the binding isotherms represented in Figs 2–4. As evident from the given figures, the dependences of B vs log$F$ (the Bjerrum plots) indicate that even at the highest drug concentration used ($F = 5.0 \times 10^{-4}$ mol l^{-1}), i.e. when the ligand to macrobiomolecule molar ratio is approaching

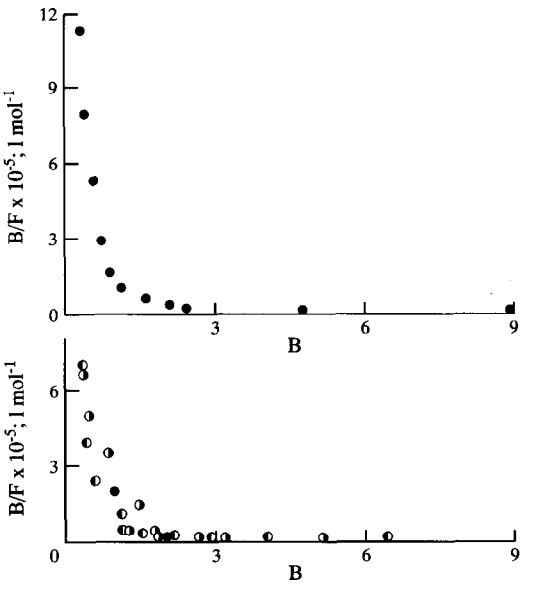

Figure 1
Scatchard plots of the reversible binding interaction between RS- (●), R- (◑), and S-propafenone (◐) and the human α_1-acid glycoprotein.

the value 50, no saturation tendency of the protein binding sites can be detected.

The affinity spectrum (the Tobler and Engel plot) of reversibly interacting RS-propafenone with the human α_1-AGP (see Fig. 2) indicates as the most adequate description of the equilibrium binding data such an isotherm which is given by equation (2), with $N = 2$, a simultaneous manifestation of the non-specific, unsaturable, binding term, and with $k_0 = 0$. However, this result of binding data computer analysis of the system comprising the human α_1-AGP and propafenone racemate could lead to the interpretation that the generated affinity spectrum recognized a simultaneous manifestation of two different reversible interactions between the given protein and individual propafenone enantiomers. (The system containing a chiral macrobiomolecule, such as α_1-AGP, and a drug which is the mixture of two enantiomers has in fact three components.)

This thesis is fully confirmed by the affinity spectra of both individual drug enantiomers, i.e. R- and S-propafenone interaction with the human α_1-AGP, as shown in Figs 3 and 4. Contrary to the picture which can be seen in Fig. 2, it is evident that for the binary mixtures, i.e. one single drug enantiomer plus the protein, the binding isotherms should be described exactly by equation (2), with the number of the classes of specific, saturable,

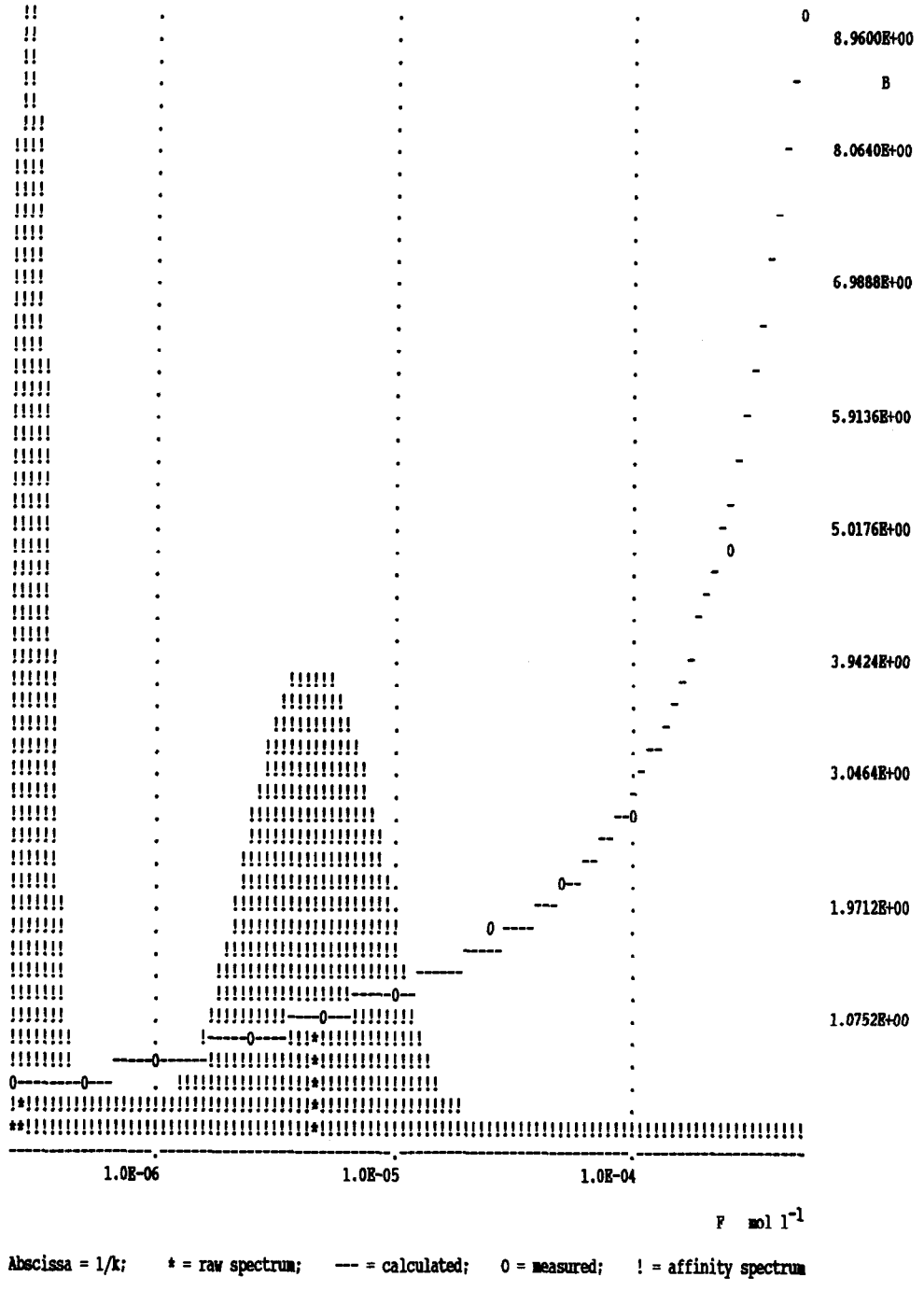

Figure 2
Affinity spectrum of the reversible binding interaction between RS-propafenone and the human α_1-acid glycoprotein. (The Bjerrum plot (----0----) of the data is included.)

binding sites $N = 1$, and the k_0 value being equal to zero.

To further treat the interaction of the R- and S-propafenone enantiomers with the human α_1-AGP, the binding parameters were computed by a non-linear curve-fitting procedure [16] using equation (2), with $N = 1$, and $k_0 = 0$. As starting parameters for the iteration the values provided by the affinity spectra of the systems containing a single enantiomer (see Table 1; also Figs 3 and 4) were applied. The starting guess value used for $n'k'$ was zero. On fitting the experimental data by non-linear regression computer analysis a very good

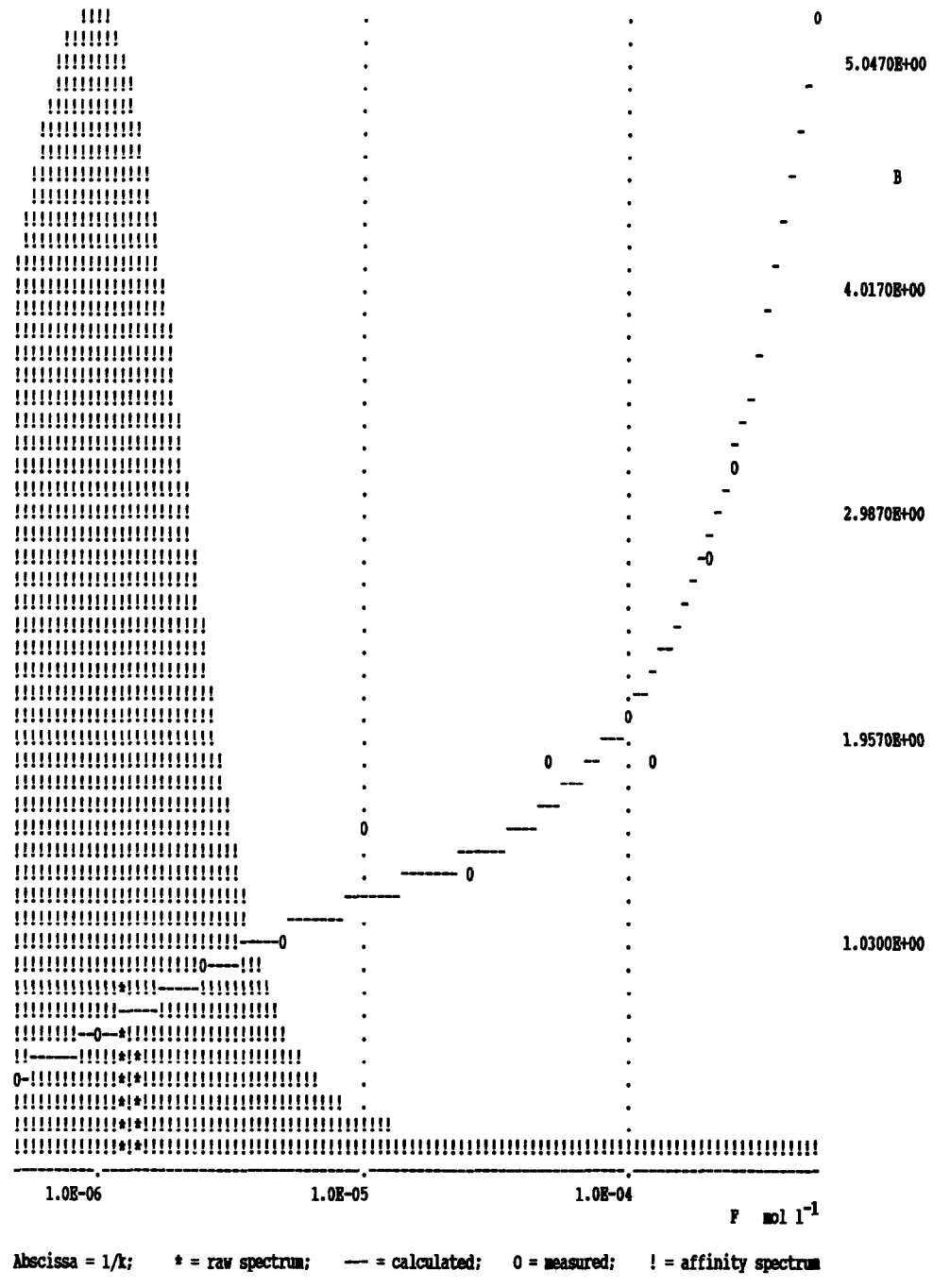

Figure 3
Affinity spectrum of the reversible binding interaction between R-propafenone and the human α_1-acid glycoprotein. (The Bjerrum plot (----0----) of the data is included.)

stability of iterations was found yielding one invariably optimal n, k, and $n'k'$ value as shown in Table 1 as means ± S.E.M. Although the difference is not too large, S-propafenone was found to bind more strongly to the human α_1-AGP than that did its R-optical antipode (the mean values of $k_S > k_R$; $(nk)_S > (nk)_R$; and $(nk + n'k')_S > (nk + n'k')_R$).

Discussion

The binding interactions between propafenone (RS-, R-, or S-) and the human α_1-AGP are herein analysed by three different mutually supplementary approaches. Whilst the observed curvilinearity of all three Scatchard plots shown in Fig. 1 exclude the

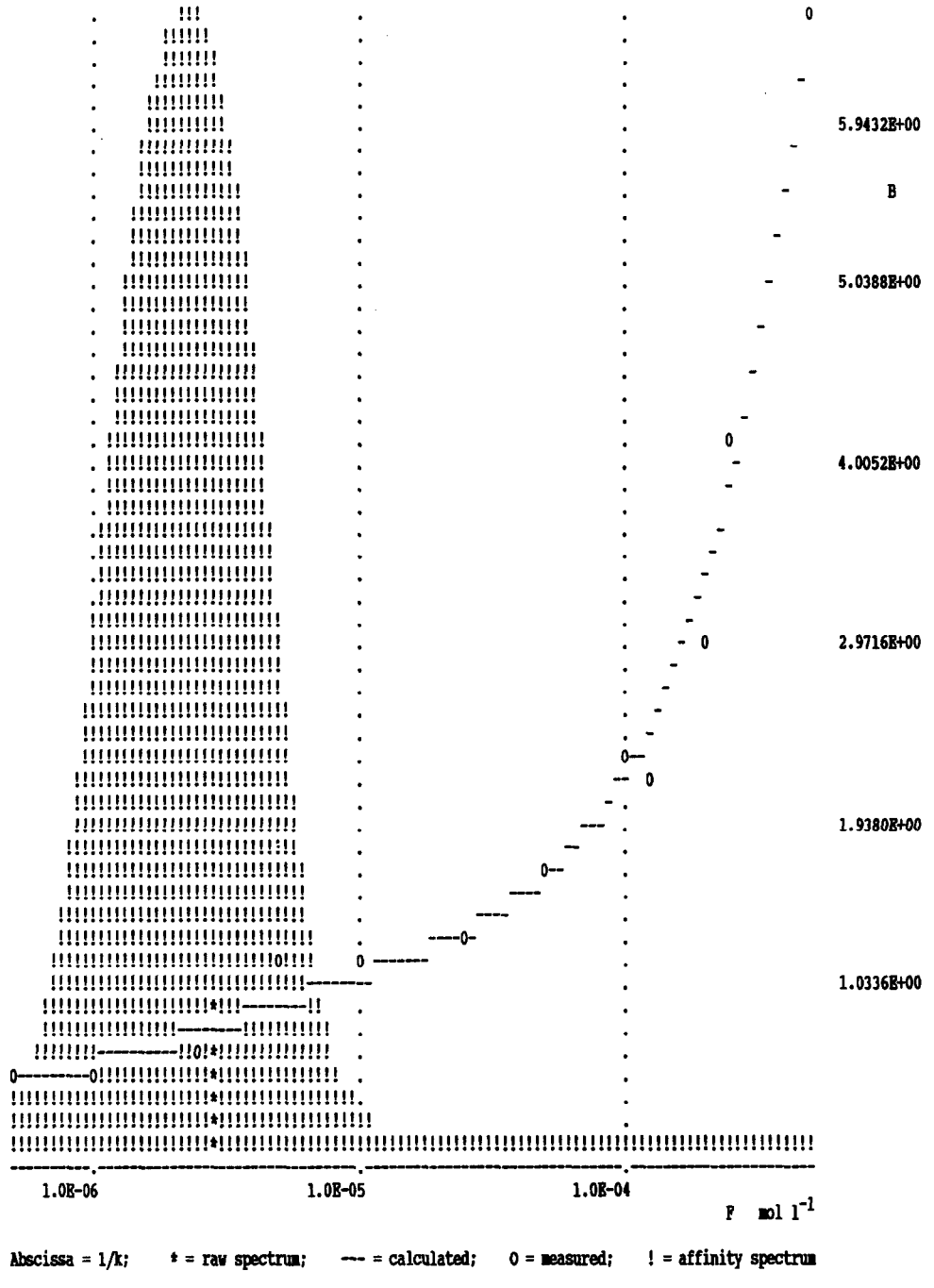

Figure 4
Affinity spectrum of the reversible binding interaction between S-propafenone and the human α_1-acid glycoprotein. (The Bjerrum plot (----0----) of the data is included.)

model of one single class of specific binding sites, i.e. equation (1) being applied for the data evaluation, the Bjerrum plots, B vs log$F$ (Figs 2–4) support the adequacy of the term $n'k'F$, i.e. a simultaneous non-specific, unsaturable, binding interaction being taken into account. [It should be stressed again that neither of the isotherms represented in Figs 2–4 indicate signs of approaching saturation, even at the highest ligand concentration set in the eluent ($F = 5.0 \times 10^{-4}$ mol l^{-1}) when the drug to injected protein molar ratio is 50.]

Thus combining the features of the Scatchard and Bjerrum plots at fulfilling the condition of performing binding measurements at the highest attainable value of the drug to

Table 1
Reversible binding parameters for the interaction of propafenone R- and S-enantiomers with human α_1-acid glycoprotein

Binding parameters*		Propafenone R-	Propafenone S-
Iteration starting value			
n		1.17	0.78
k	[1 mol^{-1}]	<7.02, 8.07> × 10^5	3.50 × 10^5
Optimized value			
n		1.33 ± 0.09	0.98 ± 0.08
k	[1 mol^{-1}]	(6.2 ± 0.94) × 10^5	(9 ± 1.88) × 10^5
$n'k'$	[1 mol^{-1}]	(6.9 ± 0.72) × 10^3	(1.07 ± 0.09) × 10^4
nk	[1 mol^{-1}]	8.246 × 10^5	8.82 × 10^5
$nk + n'k'$	[1 mol^{-1}]	8.315 × 10^5	8.927 × 10^5

*Parameters given as mean or mean ± s.e.m. values.

macrobiomolecule molar ratio, one can conclude that the valid reversible binding isotherm describing the systems studied consists of a single specific and a non-specific binding term: $B = nkF/(1 + kF) + n'k'F$. This relationship is the simplest possible of those which are applicable for fitting the binding data given in Fig. 1. Moreover, it is valid for the situation when more than one specific class of binding sites is manifest and when within the investigated concentration range no saturation is achieved.

The position of the bell-shaped curves in the affinity spectra valid for the R- and S-propafenone enantiomers (see Figs 3 and 4) differ mutually and, moreover, they are at variance with those found for the drug racemate interaction with the protein investigated (Figs 2–4). It might be tempting to assume that in the case of the propafenone racemate the two enantiomers mutually compete for the same specific class of the binding sites on the human α_1-AGP. However another, and presumably the most relevant, explanation could be that in the three-component system, i.e. the chiral macrobiomolecule (P) and the two drug enantiomers (L_1, L_2), the following equilibria are simultaneously involved:

$$P + L_1 \underset{1/k_1}{\overset{k_1}{\leftrightarrow}} P\text{–}L_1$$

$$P + L_2 \underset{1/k_2}{\overset{k_2}{\leftrightarrow}} P\text{–}L_2$$

and thus, in such a situation any application of equations (2) and (1), is in fact incorrect.

Although the higher order structure of the human α_1-AGP, as well as the "topogram" of its binding site(s) are not yet known in detail, so far only one single class of the binding sites has been considered which manifests a slight stereoselectivity predominantly at the interaction with the basic drug enantiomers [2]. In the case of propafenone enantiomers it has been shown that its S-isomer binds somewhat more strongly to plasma proteins than its R-optical antipode (unbound R-fraction = 0.076; S– = 0.049 [6]).

Similarly to the above data, the relative difference of the total affinities, found by applying the non-linear regression analysis for the two R- and S-propafenone enantiomers interacting with the human α_1-AGP (also see Table 1),

$$|(nk + n'k')_S - (nk + n'k')_R|/(nk + n'k')_S = \pm 6.86\%$$

does neither support nor exclude two possibilities: (i) that the two isomers would interact with the same class of binding sites on the protein; and (ii) that at studying the system comprising the propafenone racemate and the human α_1-AGP this protein recognizes the enantiomers in the mixture [12].

Finally, the reader's attention is drawn to the informational value of the data on the reversible binding of the racemic propafenone with the human α_1-AGP. In full agreement with the present results, Gillis *et al.* [8], employing the method of equilibrium dialysis, found a curvilinear Scatchard plot for the RS-propafenone interaction with human α_1-AGP. The authors suggested the most relevant binding model was that of two different classes of specific, saturable, binding sites: $n_1 = 0.20$, $n_2 = 0.79$. Their

data, however, do not clearly show that saturation of binding sites actually occurred at $n_1 + n_2 = 0.99$. Although the shortcomings of the Scatchard plot analysis have been discussed [12, 17, 18], numerous papers still do appear reporting drug–protein binding parameters extracted exclusively from such plots. The frequently observed curvilinearity of the graph is interpreted just as a proof for the presence of two (or more) classes of specific, saturable, binding sites on the protein examined.

On the basis of the authors' experience over several years [10, 12, 19, 20] they are now confident that in the case of evaluating the interaction of a drug mixture, such as a racemate, with the protein examined, the observed binding data have not to be further "mathematically manipulated". For a graphical illustration, the maximal admissible operation is data presentation in the form proposed by Scatchard, Bjerrum, and possibly also by Tobler and Engel (see Figs 1–4). If the model covering reversible bimolecular interaction of ligand molecules and sets of independent non-interacting binding sites on the counter-part macrobiomolecule [11] is to be applied at all, one must realize the actual meaning of the numerical values of F_i and B_i, particularly in the case of a racemate, representing a mixture of enantiomers. In such a situation two different sets of free and bound fractions corresponding to the R- and S-enantiomers, respectively, should be taken into account. The authors are, however, convinced that for an exact experimental design exclusively pure enantiomer vs protein interaction data are to be further mathematically processed. It is essential that binding data be treated as numerical values whose mode of generation must observe the fact that enantiomers may and do act as two different compounds.

In conclusion, both R- and S-propafenone enantiomers interact with almost the same total affinity to the human α_1-acid glycoprotein macrobiomolecule. Caution is necessary when the racemate is being measured and evaluated. There are no doubts about the usefulness of studying both enantiomers and their racemic mixture in binding experiments. However, a more complex data analysis is required than that of simple graphical analysis (usually just only the Scatchard plot) followed by nonlinear regression.

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