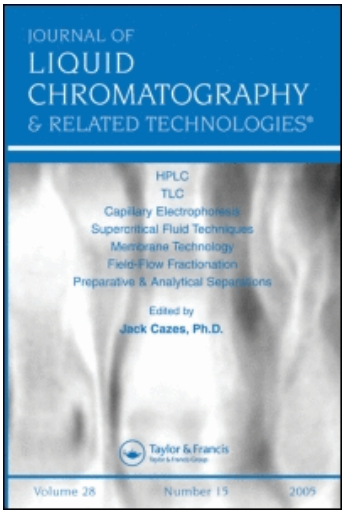


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# Study of Macrobiomolecule-Ligand Interactions by Liquid-Chromatographic Separation Methods Under Equilibrium and Nonequilibrium Conditions

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# STUDY OF MACROBIOMOLECULE-LIGAND INTERACTIONS BY LIQUID-CHROMATOGRAPHIC SEPARATION METHODS UNDER EQUILIBRIUM AND NONEQUILIBRIUM CONDITIONS

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**ABSTRACT**

The interaction of propranolol with pure human $\alpha_1$-acid glycoprotein as well as that of bilirubin with complete human serum was studied using high-performance liquid chromatography. Hummel and Dreyer (equilibrium) size exclusion as well as (nonequilibrium) zonal elution chromatographic methods were applied. The binding interaction data were evaluated according to three different approaches introduced by Scatchard, Bjerrum, and by Tobler and Engel.

Procedure schedules designed for the study and evaluation of the reversible (equilibrium) macrobiomolecule-ligand interaction are discussed and critically commented.

## INTRODUCTION

Binding interactions of a macrobiomolecule with a ligand resulting in the development of a complex, an associate, represented by e.g. an enzyme-substrate, antigen-antibody, receptor-hormone, have been the subject of studies in several (bio-)chemical, biological, medical, and interdisciplinary branches [1, 2].

The binding parameters of "weakly" interacting components, typical examples of which are protein and drug containing systems, forming a homogenous liquid phase have been most frequently determined by using the method of equilibrium dialysis. The binding characteristics of strongly interacting components in "heterogenous" systems have been predominantly studied by membrane (ultra-)filtration.

This paper presents and comments the results of HPLC studies of two interacting systems, i.e.

- human $\alpha_1$-acid glycoprotein with propranolol, and
- human serum with bilirubin.

The commentary addresses methodological questions, focusing on the classification of the analytical HPLC methods used, as well as on the procedures applied for the evaluation of the experimental data.

## MATERIALS AND DATA ANALYSIS

### Ligands and Chemicals

Propranolol racemate (ICI Pharmaceuticals, Macclesfield, Great Britain), bilirubin *p.a.* (Merck, Darmstadt, Germany).

Dithioerytritol 99% (DTE; Aldrich-Europe, Beerse, Belgium). $KH_2PO_4$, $Na_2HPO_4$ x 12 $H_2O$, NaOH, all of *p.a.* purity grade (Merck, Darmstadt). The water used was of Millipore Q quality (Millipore Corporation, Bedford, Ma, U.S.A.).

## Proteins and Biological Samples

Human $\alpha_1$-acid glycoprotein ($\alpha_1$-AGP; Op.-Nr. 8376, Behring Institute, Germany), human serum albumin (HSA; No. A 1887, Sigma Chemical Co., St. Louis, Mo, U.S.A.).

Nonhemolyzed serum of a healthy adult (AS; 2.9 mg of the total bilirubin per liter) and sera of two newborn infants ($IS_1$, $IS_2$; 129 mg/l and 102 mg/l, respectively) were from the Centre Hospitalier Intercommunal de Créteil, France.

## Working Solutions

The bilirubin working solution was prepared by dissolving 20 mg of bilirubin in 3 ml of aqueous NaOH (0.1 mol/l). The volume of the solution, to which 30 mg of DTE was added, was supplemented to 10 ml by phosphate buffer solution (0.01 mol/l, pH 7.4).

The blank solution was prepared by the same way, but without bilirubin.

## Working Serum Samples

Samples for the HPLC analysis were prepared by mixing the bilirubin working solution and the blank solution with the serum. In the analyzed set of samples, coded/marked from a to m, the dilution factor of the serum was 0.6. (The volume of the injected sample, 10 µl, thus consisted of 6-µl of native serum and of 4-µl of the diluent.)

The concentration of exogenous bilirubin in the diluent for the preparation of a-m samples was: 0, 0.0625, 0.125, 0.1875, 0.25, 0.3125, 0.375, 0.5, 0.625, 0.75, 1, 1.5, and 2 mg/ml.

## Scatchard Plot

The binding isotherm of a ligand interaction exclusively with one 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 [3] is frequently used for the graphical evaluation of binding of the drug to the receptor. The linear dependence B/F *vs.* B, simply called also "Scatchard plot", has the following characteristics [4]:

- 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. logarithm F [2] - proved to be especially advantageous for the assessment of the saturability of the examined binding interaction [4-6]. The B *vs.* logF dependence has also some characteristic features [4]:

- 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.



## RESULTS

Figure 1, record c, represents the chromatogram of the given amount of $\alpha_1$-AGP dissolved in the eluent, i.e. in phosphate buffered propranolol solution. The first, positively oriented, peak belongs to the protein-drug complex. The second peak, with opposite orientation, signalizes the drug deficit in the analyzed sample caused by the binding of propranolol to $\alpha_1$-AGP.

The amount of the drug fraction bound to the protein was determined by the internal calibration technique [7-11] on the basis of HPLC analysis of the set of samples which are characterized by the constant $\alpha_1$-AGP concentration but

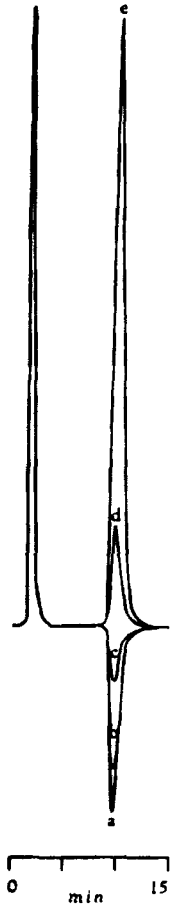


FIGURE 1. Chromatograms of human $\alpha_1$-AGP (0.6 g/l) dissolved in phosphate buffered solution (0.067 mol/l, pH 7.4) of propranolol, with the drug concentration of $1.0 \times 10^{-5}$ (a), $2.5 \times 10^{-5}$ (b), $5.0 \times 10^{-5}$ (c), $1.0 \times 10^{-4}$ (d), and $2.5 \times 10^{-4}$ (e) mol/l.

Column: 4.7 mm x 15 cm
Column temperature: 37.0 °C
Sorbent: LiChrosorb Diol, 10 µm
Eluent: Phosphate buffered solution (0.067 mol/l, pH 7.4) of propranolol with the concentration of $5.0 \times 10^{-5}$ mol/l
Flow rate: 1 ml/min
Volume of the injected sample: 25 µl
Detector: Spectrophotometer 450; Abs. = 291 nm

by variable propranolol concentrations (figure 1, records a-e). For example, on using this procedure for the set propranolol concentration in the mobile phase F = 5.0 x $10^{-5}$ mol/l the corresponding B value was found to be 1.31 (see figure 2, panel b). On changing the experimental conditions, i.e. at various drug-concentrations set in the eluent, for each selected $F_i$ value its corresponding $B_i$ value was determined.

Figure 2, panels a) and b), shows the binding interaction of the human $\alpha_1$-AGP with propranolol as discrete points plotted in the form of B/F *vs.* B as well as B *vs.* F. As evident from panel a), it is impossible to fit the experimental points by a straight line, and thus conclusively, $\alpha_1$-AGP does not interact with propranolol exclusively within one single class of specific, saturable, binding sites.

Figure 3, record a, represents the chromatographic elution profile of the diluted native serum of a healthy adult. The HPLC records b-m show the distribution of the gradually increasing addition of the pigment - exogenous bilirubin - to the examined AS serum sample. The peak eluted first represents β-lipoprotein "stained" with the increasing amount of exogenous pigment; the following peak belongs to the albumin-bilirubin complex. On the records g-m, the plateaus indicate the presence of a free/dissociated pigment fraction in the analyzed samples, most probably due to the saturation of the capacity of serum albumin and β-lipoprotein to bind bilirubin.

Figure 4, record a, shows the chromatogram of the diluted native infant serum ($IS_2$). Contrary to the healthy adult serum sample with a similar total bilirubin concentration (cf. figure 4, record a, and figure 3, record c or d), the HPLC analysis of the native $IS_2$ sample exhibited a significant amount (plateau) of the free/dissociated endogenous pigment fraction. As evident from the records b-m (figure 4) the increasing amounts of exogenous bilirubin added to the $IS_2$ sample are virtually

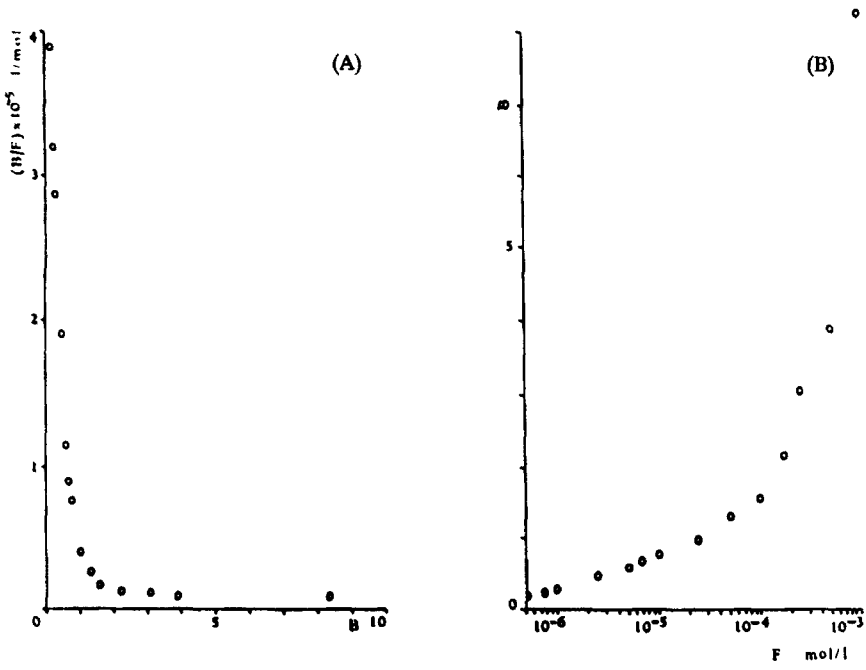


FIGURE 2. Binding interaction of human $\alpha_1$-AGP with propranolol.



proportionally distributed between the bound and free pigment fraction.

## DISCUSSION AND COMMENTARY

The interaction of a macrobiomolecule, a protein (P), with a low-molecular-weight compound, a ligand (L), can be generally expressed by the following chemical equations -

$$P + L \rightarrow PL \qquad (2),$$

$$P + L \leftrightarrow P\text{-}L \qquad (3).$$

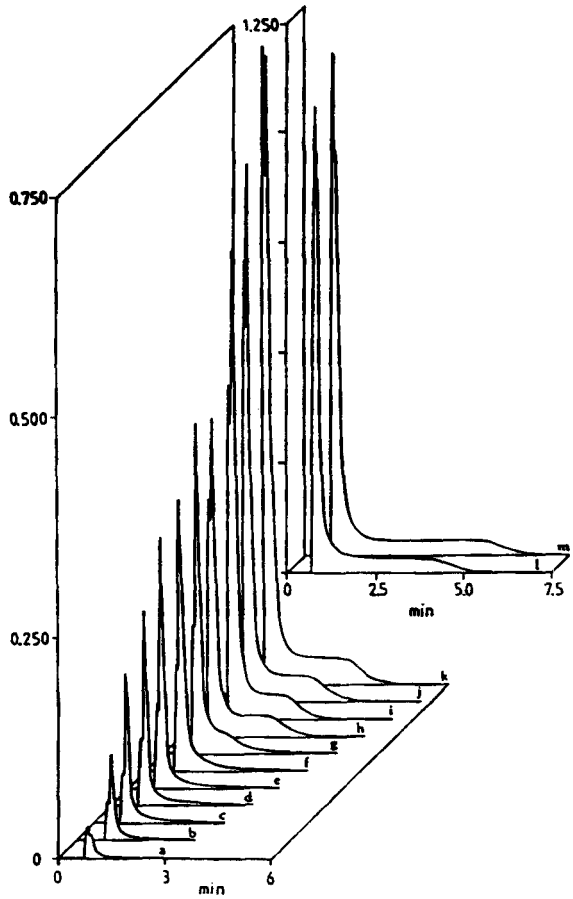


FIGURE 3. Chromatograms of serum of a healthy adult (AS) with the total bilirubin concentration in individual samples: 1.74 (a), 26.74 (b), 51.74 (c), 76.74 (d), 101.74 (e), 126.74 (f), 151.74 (g), 201.74 (h), 251.74 (i), 301.74 (j), 401.74 (k), 601.74 (l), and 801.74 (m) mg/l.

Eluent: Phosphate buffered solution (0.067 mol/l, pH 7.4) with the concentration of 80 mg HSA/l and with the addition of the bilirubin working solution = 1000:0.05
Column: 4.7 mm x 15 cm
Sorbent: LiChrosorb Diol, 5µm
Flow rate: 1 ml/min
Detector: Filter photometer 440; Abs. > 436 nm

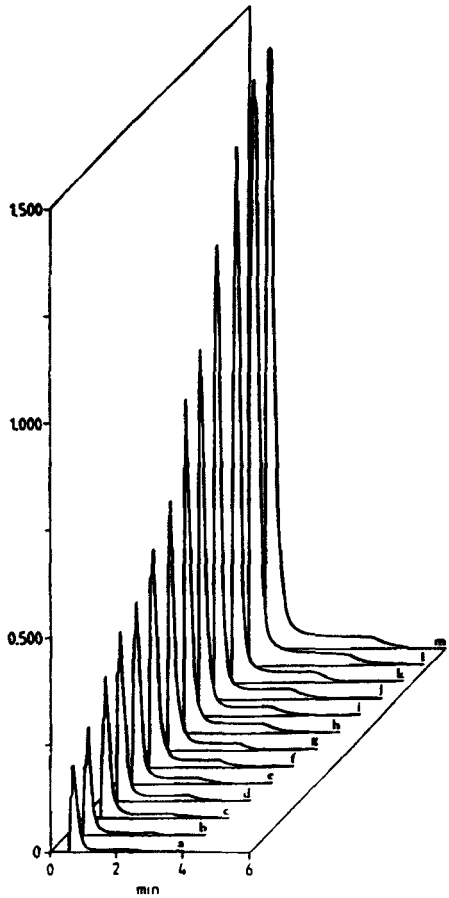


FIGURE 4. HPLC elution profiles of infant serum ($IS_2$) with the total bilirubin level in individual samples: 61.2 (a), 86.2 (b), 111.2 (c), 136.2 (d), 161.2 (e), 186.2 (f), 211.2 (g), 261.2 (h), 311.2 (i), 361.2 (j), 461.2 (k), 661.2 (l), and 861.2 (m) mg/l.

Eluent: Phosphate buffered solution (0.067 mol/l, pH 7.4) with the concentration of 2 ml AS/l and with the addition of the bilirubin working solution = 1000:0.05

(Column, sorbent, flow rate, detector - see figure 3.)

Equation (2) characterizes an irreversible reaction where a qualitatively new substance - the product PL - is formed from the reactants P and L. In living organisms such a (bio-)chemical reaction, diminishing the endogenous protein and simultaneously generating a new xeno-macrobiomolecule PL, characterizes usually an adverse pathophysiological or even pathological process.

Finding of a PL-type substance, e.g. in human blood, can sometimes directly serve as an indicator of a certain disease. Besides sugar in urine, the diagnosis of *diabetes mellitus* is indicated also by glycosylated albumin in blood plasma.

In *in vitro* experiments, the stechiometry of the reaction expressed by equation (2) is studied usually on using a radioisotope labeled ligand ($L^*$). The ternary mixture of the reactants P and $L^*$ and the product $PL^*$ is separated only into two fractions a low-molecular of the nonconverted ligand $L^*$ and a macromolecular one which is a binary mixture of the protein P and the substance $PL^*$.

For quick and efficient separation of two fractions differing in their molecular weights the HPLC method working under size exclusion chromatographic mode is being primarily applied. The question of the quantification of the amount of the nonconverted ligand and of the generated product is, thanks to the application of the labeled reactant $L^*$, simplified to measuring the radioactivity of the two fractions, i.e. $PL^*$ + P, and L* [12].

Unlike the chemical reaction described by equation (2), which in the living body represents adverse processes, the reversible interaction expressed by equation (3) involves several processes which are essential for physiological functioning of the organism. Many such reversible interactions of effector molecules (hormone, substrate, low- or high-molecular-weight antigen) with corresponding macrobiomolecules (receptor, enzyme, antibody) do not only constitute the basis of biochemical regulation, signalization and defense of particular animal cells, but

also the basis of (bio-)chemical "communication" between cells in the whole body.

However, the quantitative stechiometric analysis of the reaction described by equation (3) is a very complicated task. This difficulty results from the demand to determine the instantaneous molar concentrations (molar activities [P], [L], and [P-L]) of each component of the ternary mixture without deranging the equilibrium described by equation (3) and from the fact that the "product" P-L is a complex, an associate, which actually does not exist in the form of a pure, isolated chemical item.



## The Method of Hummel and Dreyer

In 1962 Hummel and Dreyer were first to describe [7] a liquid chromatographic separation method which has been applied in studying reversible interactions between a macrobiomolecule and a ligand [10, 13-18]. The characteristic feature of the technique developed by Hummel and Dreyer is the equilibration of the chromatographic column packing with the mobile phase which contains a given ligand (drug) concentration. (The most frequently used column fillings are sorbents which work in the mode of size exclusion chromatography or in the separation mode based on ion-exchange effects. The major requirement for the applicability of a particular sorbent is its "inertness" against the injected sample containing a protein). The injected, analyzed sample is a certain amount of the macrobiomolecule dissolved in the eluent.

The injection of such a sample usually yields two peaks in the resulting chromatographic record (see figure 1, record c). The first (positive) peak belongs to the protein-drug complex. Its retention volume usually equals the void volume of the chromatographic column used. The second, negatively oriented peak, which is detected at the retention volume of the drug, manifests the ligand deficit

in the analyzed sample caused by the association of a particular drug fraction with the given amount of the protein.

The amount of the protein-bound drug fraction is determined by the internal (see figure 1) or external calibration technique [19]. The purpose of the use of either calibration technique is to find such a concentration of the ligand which just compensates the drug deficit in the analyzed sample, or to determine that ligand excess which eliminates the appearance of the second peak on the chromatographic record.

The rationale of the Hummel and Dreyer method is that the rate of the stabilization of the composition of the protein-ligand complex injected into the eluent stream containing the given concentration of the free drug is much higher than the speed of the chromatographic process itself [18]. Since in practice this condition is fulfilled usually *a priori*, the Hummel and Dreyer method is generally declared to be an equilibrium liquid chromatographic separation method for studying reversible macrobiomolecule-ligand interactions.

However, despite the fact that the number of the ligand moles bound by one mole of the protein (B) depends exclusively on the drug concentration set in the eluent (F), the gradual dilution of the protein-ligand complex, which naturally occurs during the chromatographic process, can significantly influence the macrobiomolecule-drug interaction equilibrium. As a result of this process, the determined binding parameters are only apparent/"pseudoequilibrated" [20].

Another critically not yet evaluated characteristic feature of any liquid chromatographic study of a reversibly interacting system, including the method introduced by Hummel and Dreyer, is a pressure drop along the chromatographic column which may result in a permanent change of the protein-drug complex composition when it is transported through the column body.

## Binding Data Analysis

The reversible interaction of a ligand with an acceptor macrobiomolecule is generally described by the following equation [3, 21] -

$$B = \sum_{i=1}^{N} n_i k_i F/(1 + k_i F) \qquad (4),$$

where $n_i$ represents the number and $k_i$ the affinity constant of the i-class of binding sites for which the following limits are simultaneously valid:

- all binding sites on the macrobiomolecule exist independently of the presence or absence of the ligand in the system and, due to the interaction of the two components, the binding sites do neither arise nor disappear,

- each binding site on the macrobiomolecule interacts with the ligand independently.

Yet the validity of the above mentioned limiting conditions, and thus the aplicability of the dependence (4) called also Scatchard equation, is equivocal since neither the plasmatic protein nor the receptor protein exist in the system in a "frozen" conformation carrying pre-existing classes of binding sites characterized by fixed affinities for the given ligand [22]. Moreover, the evaluated constants have only a pseudovalue [2, 23] because the solution of equation (4) is not an unambiguous, objective, set of values of $n_i$ and $k_i$ pairs.

Therefore, on comparing binding data from various laboratories it is recommended to consider the shape and the position of the binding isotherms and not the numeric values of the binding constants [23].

## The Tobler and Engel Plot

In 1983 Tobler and Engel published an original procedure of computer analysis of equilibrium binding

interactions, the result of which is the so-called Affinity spectrum [24]. This spectrum (plot) shows the number of binding sites *vs.* corresponding dissociation constants ($1/k_i$).

The input data are represented exclusively by the set of values $F_i$ and $B_i$. The evaluation based on the linear programing principle results in pairs $n_i$ and $1/k_i$, the values of which can be read from the given plot both visually and in the form of numeric data [24].

The affinity spectrum (Tobler and Engel plot) of the system containing $\alpha_1$-AGP and propranolol (see figure 5) indicates as the most adequate description of the binding data the isotherm in the following form -

$$B = \sum_{i=1}^{N} n_i k_i F/(1 + k_i F) + n'k'F \qquad (5)$$

whith the number of mutually independent classes of specific binding sites N = 2, while the product n'k'F characterizes the simultaneous manifestation of nonspecific (unsaturable) binding. But this result of binding data computer analysis of the system comprising human $\alpha_1$-AGP and propranolol racemate lends itself also to the interpretation that the generated affinity spectrum allowed to recognize the simultaneous manifestation of two different reversible interactions between the given protein and individual propranolol enantiomers. (The system containing the chiral macrobiomolecule, $\alpha_1$-AGP, and the drug which is the mixture of two enantiomers has in fact three components.)

## Binding Interaction of Human $\alpha_1$-AGP with (+)- and (-)-Propranolol

Despite the fact that the (-)-propranolol enantiomer is pharmacologically up to 100-times more effective than its

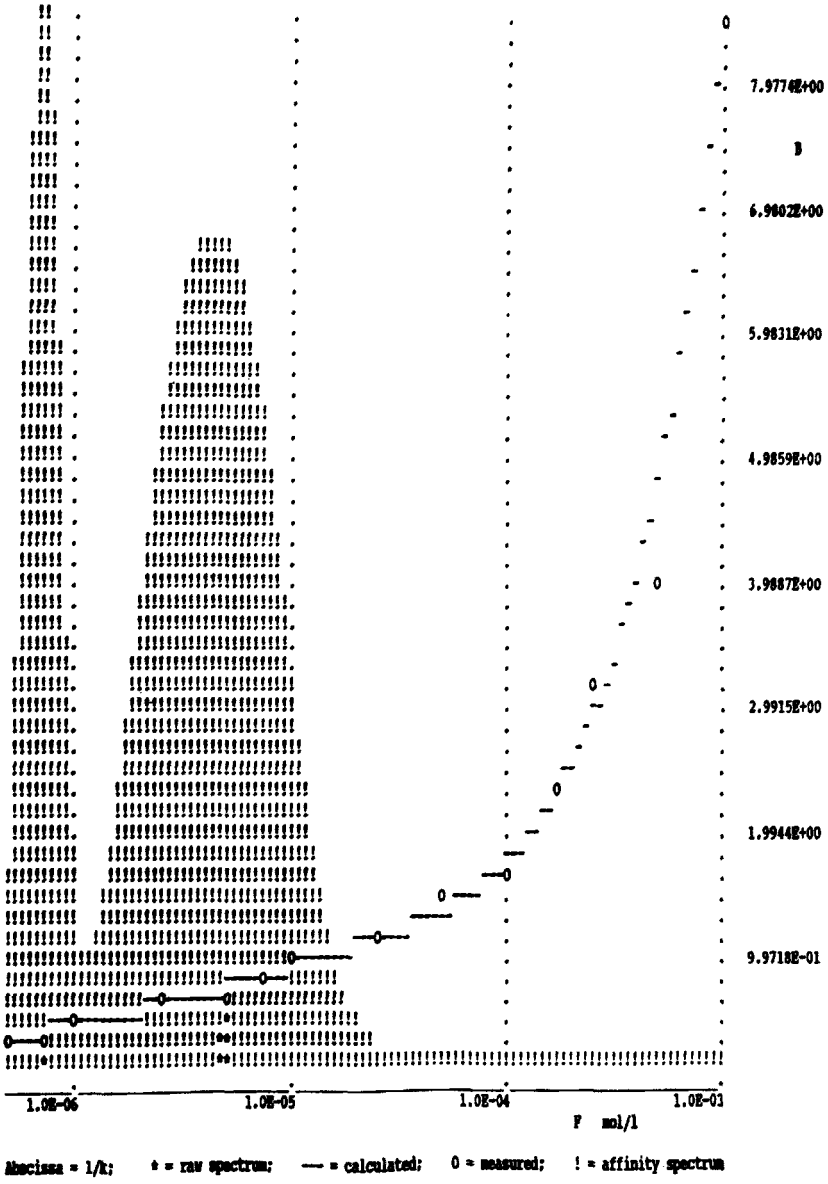


FIGURE 5. Tobler and Engel plot (affinity spectrum) of the binding interaction between human $\alpha_1$-AGP and propranolol.

(+)-isomer, in therapeutic practice this β-adrenoceptor blocking drug is administered in the form of a racemic mixture of both its optical antipodes. Thus propranolol racemate was used in the majority of determinations concerning the interaction of this β-adrenolytic even with individual proteins [10, 25-30].

So far only few laboratories have been studying the binding interactions of individual propranolol enantiomers, using either complete blood plasma from various animals (human, dog, rat) or also some isolated and purified plasma proteins ($\alpha_1$-AGP, HSA) [31-35]. However, due to the lack of any exact knowledge about the stereospecifity of the reversible interaction of macrobiomolecules with individual propranolol enantiomers at molecular level, the results published so far can be classified as relatively highly inconsistent. The value and relevance of the stated binding parameters is also doubtful, both due to the use of not standardly pure proteins (e.g. $\alpha_1$-AGP) and mainly due to the evaluation of experimental data by using non-adequate mathematical description of the corresponding binding isotherm.



Reversible interactions in both systems containing standardized human $\alpha_1$-AGP with the particular (+)- or (-)-propranolol enantiomer have been characterized only in the senior author's Laboratory. For these measurements the HPLC method according to Hummel and Dreyer was used [36, 37].

By using this method and by combining the processes of the experimental data analysis according to Scatchard, Bjerrum, as well as to Tobler and Engel, the determined binding interaction between $\alpha_1$-AGP and (-)-propranolol was found to be saturable, with the parameters $n = 0.81$ and $k = 2.73 \times 10^5$ l/mol. The binding isotherm of the system $\alpha_1$-AGP and (+)-propranolol was adequately described by the interaction parameters $n = 0.38$, $k = 3.39 \times 10^6$ l/mol, and by the value of $n'k' = 1.39 \times 10^4$ l/mol.

The measurement of circular dichroism in both systems, i.e. human $\alpha_1$-AGP with (-)- or (+)-propranolol enantiomer [36], confirmed the adequacy of evaluating the interaction between the protein and each individual isomer with the use of different binding isotherms expressed by equation (1), or equation (5) with the value of $N \approx 1$.

## Retention Analysis with Zonal Elution Chromatography

Qualitative as well as quantitative evaluation of the interaction between a macrobiomolecule and a low-molecular-weight ligand can be done also on the basis of measuring the kinetics of the protein-ligand complex dissociation by the method of zonal elution chromatography [11, 38, 39]. On using this technique, a certain amount of the sample - the solution of the protein plus ligand - is injected into the chromatographic column eluted with the solvent of the analyzed sample, most frequently phosphate buffer. For the separation of the sample components the mode of size exclusion, ion-exchanging, affinity chromatography, *etc.* is used.

In general, the protein-ligand complex (together with the protein surplus) is eluted from the column first followed by another peak belonging to the free, non-associated ligand. However, it is also necessary to emphasize that in zonal elution chromatography the protein-ligand complex, while transported through the column, continually dissociates both because of its constant mixing and gradual dilution by the eluent and because of the ligand retardation by the stationary phase. (The disintegration process of 10 % of the complex characterized by the association constant value $k = 10^{10}$ l/mol lasts for about 1000 seconds; when $k = 10^9$ l/mol this process lasts only 100 seconds, etc.)

Besides the above mentioned facts, however, the method of zonal elution chromatography is primarily used in

studying "solid" complexes, typical examples of which are antigen-antibody, enzyme-substrate, etc. The selection of zonal elution chromatography is favored mainly by the fact that this technique works effectively also with minute amounts of material usually available in immunology, enzymology, and in many other scientific fields and medical branches, as e.g. in pediatrics.

## Interaction of Human Serum with Bilirubin

The serum capacity to bind bilirubin has to be determined very often in pediatrics in assessing the course of neonatal jaundice. One of the methods for determining the reserve bilirubin binding capacity of the infant serum is based on the sample "filtration" through the Sephadex G-25 minicolumn bed. The bilirubin fraction firmly bound to the proteins passes through the column while the fraction of "free" (loosely bound) pigment remains adsorbed onto the packing material. This free/dissociated bilirubin fraction is eluted from the minicolumn with the help of an alkaline solution, in the next step the pigment is extracted into the chloroform and finally quantified spectrophotometrically.

The serum reserve bilirubin binding capacity is thus determined by titrating the sample with exogenous bilirubin and by the subsequent determination/demonstration of a free pigment portion. By using a similar filtration technique through Sephadex G-100, Cooke and Roberts found [40] that the exogenous bilirubin added to the adult serum associated with two proteins - with albumin and β-lipoprotein.

On applying this method of filtrating a bilirubin stained sample through the gel bed the following important phenomena should however be taken into account:

- The molecular weight of the β-lipoprotein ($\approx 2 \times 10^6$ Da) is significantly higher than that of albumin (66210 Da [41]). This is why at gel filtration of stained as well as

not-stained proteins β-lipoprotein is first eluted from the sorbent, followed by albumin.

- The Sephadex type adsorbent has a relatively high affinity to bilirubin. (After application of the stained β-lipoprotein, the Sephadex remains colored as the result of both the process of complex dissociation and of adsorption of the dissociated bilirubin portion by the sorbent [40].)

- Because of a certain albumin reserve capacity to bind bilirubin, this protein, eluted after the β-lipoprotein, desorbs/extracts a part of the pigment adsorbed on the sorbent.

In contrast to the above mentioned gel filtration technique, the HPLC method described herein for assessing the reversible bilirubin binding to human serum proteins exploits simultaneously the mechanism of the size exclusion and (ad-)sorption chromatographic separation. Under the conditions of the presented method the dissociation of the protein-bilirubin complex was suppressed by addition of bilirubin to the mobile phase. The solubility of the pigment in the phosphate buffer solution used (0.067 mol/l, pH 7.4) was warranted by the "carrier" - HSA [42, 43] or the adult serum [43].

On using the presented HPLC method, the fractions of bilirubin associated with albumin as well as with β-lipoprotein, and also the free - loosely bound/dissociated - pigment portion (see figures 3 and 4) were determined in serum samples of an adult human and of newborn infants.

While in the native $IS_1$ serum sample the amount of the free (dissociated) bilirubin fraction represented only 11.5 %, the amount of this fraction in the $IS_2$ sample was up to 17.3 % (figure 4, record a). The titration of $IS_1$ and $IS_2$ sera with exogenous bilirubin demonstrated that in the $IS_1$ sample the binding capacity of the serum albumin was significantly higher than that of β-lipoprotein, while in the $IS_2$ sample the pigment was associated with β-lipoprotein and with albumin to a comparable extent [43].

## *ADDENDUM*

Besides the interaction of human $\alpha_1$-AGP with (+)- and (-)-propranolol [36] also reversible binding of the basic drug propafenone and its (R)- and (S)-enantiomers with $\alpha_1$-AGP [44], as well as that of the acidic drug pirprofen including its (+)- and (-)-optical antipodes with HSA [45] was studied in the senior author's Laboratory. On the basis of the acquired experimental experience, it is possible to suggest the following general design of a study procedure and the evaluation of data on interactions between a macrobiomolecule, protein, and a drug as well as its isomers/enantiomers -

- measurement of the interaction of the drug (racemate) with the given protein in an as wide as possible range of values of the [L]/[P] ratio using the HPLC method (the Hummel and Dreyer technique),

- measurement of the interaction of the given protein with individual drug enantiomers by the above mentioned technique,

- mutual comparison of binding isotherms (Scatchard as well as Bjerrum plots) of all systems measured,

- processing of the results of interaction measurement of the given protein with individual drug enantiomers by using the computer program "AFFINITY SPECTRA" [24],

- comparison of the affinity spectrum of the given system with characteristics yielded by the Scatchard and/or Bjerrum plot,

- additional determination of the binding interaction nature of the studied system containing the corresponding drug enantiomer by using the method of circular dichroism, and/or nuclear magnetic resonance [45],

- suggestion of the most plausibly valid binding isotherm of the system studied,

- mathematico-statistical processing of the experimental data by using the method of non-linear regression only if equation (1), or equation (5) with the value of N = 1 can be applied.

## CONCLUSION

The study of drug interaction with plasma proteins is an indispensable phase in the development of new drugs and in their introduction into clinical practice.

Since the molecules of almost all synthetic drugs have one or more chiral centers [46, 47] the elaborated experimental procedure of studying and evaluating the reversible interaction between a given protein and a drug as well as its isomers can be classified as an important methodological contribution.

The HPLC method of the determination of the free and the protein bound bilirubin fractions in an infant serum sample represents an efficient diagnostic tool. The endogenous and exogenous bilirubin distribution between albumin and β-lipoprotein can be completely determined by examining even a minute sample volume.



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