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Review

Macromolecule–ligand binding studied by the Hummel and Dreyer method: current state of the methodology

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Abstract

The use of the Hummel and Dreyer method to measure binding parameters of ligand–macromolecule associations is reviewed. The possibility to determine the number of binding sites and their association constants, even in the case of low affinity, and to control the free ligand concentration as an independent variable are the main advantages of the method. The conditions of the validity are rapid equilibrium kinetics, independence between ligand binding and macromolecule association, and identical retention rates between free and bound macromolecules. Initially developed on soft gels, the method has been applied to high-performance chromatography and capillary zone electrophoresis. Technical progress such as increase in resolution, detection sensitivity, and automation have improved its utilization. The binding parameters given by the Hummel and Dreyer method are in general similar to those obtained by other techniques, in comparable experimental conditions (equilibrium dialysis, ultrafiltration, frontal elution, vacancy peak method, vacancy affinity capillary electrophoresis, retention analysis, affinity chromatography and affinity capillary electrophoresis, physical methods). The choice between these methods is directed by material availability and practical constraints. Separation by new types of chromatographic columns or by capillary zone electrophoresis would enable the study of the simultaneous binding of different drugs on the same macromolecule and their competition.

Keywords: Reviews; Macromolecule–ligand binding; Hummel and Dreyer method

Contents

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1. Introduction

The study of macromolecule–ligand binding characteristics is a problem of practical interest for biology and medicine. The levels of free drugs in plasma (or their availability from a loosely bound state), on which depend the therapeutic effects, are determined by the affinities of the proteins (mainly albumin) for these drugs, and their knowledge is important for deciding their dosage. On the other hand, the saturation of biological receptors by specific small molecules (hormones, neurotransmitters) can be described by the same models.

A lot of very different methods have been developed to solve this problem. They have been already reviewed, in particular by Sebille et al. [1], Oravcova et al. [2], and Busch et al. [3]. A few of these methods involve previous separation of the constituents (filtration, precipitation, chromatography), which may disturb their mutual equilibrium. Those which do not require this step include physical methods, which make use of specific properties of the macromolecule–ligand systems (UV or IR spectrophotometry, fluorescence emission, circular dichroism, magnetic resonance), and analytical methods in which equilibrium is preserved. Among the latter, are equilibrium dialysis and different chromatographic methods, including that developed by Hummel and Dreyer [4], which is the subject of this review.

The principle of the Hummel and Dreyer method is as follows: a known quantity of a macromolecule (purified or not) is injected on a size exclusion chromatography column and eluted with a buffer containing a constant concentration of ligand. An amount of ligand, determined by the dissociation constant(s) of the equilibrium and the free ligand concentration, binds to the macromolecule and migrates with it, while a trough in the ligand concentration, corresponding to the quantity withdrawn from the solvent, migrates at its proper rate (Fig. 1). In these conditions, the macromolecule and the complex(es) remain in equilibrium with the ligand during the separation and no dissociation occurs, even in the case of weak associations.

Because of this interesting feature, this method has been used for numerous drug binding determinations in biochemistry and medicine. The most studied has been that of the anticoagulant warfarin on human (HSA) or bovine (BSA) serum albumin [1,4–9]. The affinities of several other drugs for these proteins have been determined by this method, in its original or modified form: furosemide [5,10], ceftriaxone [10,11], β_2-blocker ICI [10], phenobarbital and phenytoin [12], phenylbutazone [1], carvedilol [13], buspirone [14]. The binding of different drugs has also been investigated on glycosylated HSA [15] and on α_1 acid glycoprotein (AGP) [10,13,16].

Cation-binding capacities of particular proteins have been examined by this method: melanotropic-lipolytic peptide IIF for Ca^{2+}, Mg^{2+}, Na^{+}, K^{+} [17], calcimedins [18], calcium-binding proteins from porcine liver [19] and arrestin [20] for Ca^{2+}, BSA and bovine α lactalbumin for Sr^{2+} [21], collagen for Pb^{2+} [22].

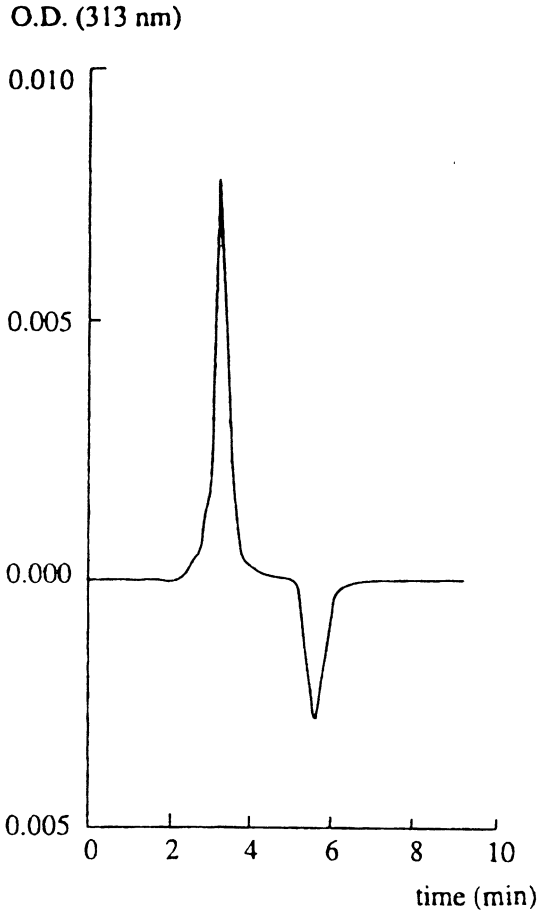

Fig. 1. Hummel and Dreyer type chromatogram obtained for a sample of 12.5 μl of HSA solution (2 g/l) in eluting solution; eluent, warfarin solution 0.5×10^{-6} *M* in phosphate buffer (pH 7.4); flow-rate, 0.5 ml/min; temperature, 37 °C; support, Glycophase G, 100 A. Reprinted from Ref. [5].

The Hummel and Dreyer method has been also applied to the study of miscellaneous equilibria:

- enzymes and substrates or inhibitors [4,23–25],
- tubulin and calmodulin [26] or antimitotic agents [27,28],
- polysaccharides and flavour compounds [29],
- cyclodextrins and vitamin B-compounds [30], steroids [31] and various drugs [32],
- lipocalin proteins and biogenic amines [33] or nucleotides [34],
- cytochrome P450 and steroids [35].

2. Theoretical aspects

According to the multiple equilibria theory, the reversible binding of a ligand on a macromolecule is governed by the equation [36,37]:

$$\bar{r} = \sum_{i=1}^{i=m} \frac{n_i K_i(A_i)}{1 + K_i(A_i)}$$

where $\bar{r}$ is the mean number of moles of ligand bound per mole of macromolecule, (A_i) the free ligand concentration, n_i the number of independent sites of class i, K_i their association constant with the ligand, and m the total number of classes.

In order to obtain valid binding parameters by this formula, the equilibria between the constituents must be continually established throughout the chromatography. As protein–ligand binding associations are very rapid, the dissociation process is generally rate limiting. It has been estimated that the half-life of the complex dissociation must be less than 5–10% of the elution time, for assumption of "instantaneous equilibration" [38]. It is the case for instance with the warfarin–albumin binding [8]. The shape of the vacancy peak can also give information on the kinetics of the dissociation: asymmetry and broadening of the peak mean that the kinetics may be too slow.

During the chromatography, the protein peak is diluted several times (3–10 times according to the conditions). Theoretically, the protein concentration does not play any role in the binding ratio. However, self-association of protein has been shown to interact, in certain cases, with protein–ligand binding [39,40]. The apparent affinity constants of certain drugs for HSA or BSA may then increase with decreasing protein concentration. We have verified that the quantity of ADP bound by the chloroplast ATPase CF1 was strictly proportional to the injected amount of sample, thus that the bound ratio remained constant with varying protein concentration [41]. In order to have constant plateaus of protein concentration, instead of peaks, Brumbaugh and Ackers [42] injected large volumes into the column. The influence of macromolecular association on small ligand binding has been analysed by Cann and Hinman from a theoretical point of view [43]. It has been concluded that the Hummel and Dreyer method was well fitted for quantitating ligand binding and guidelines have been drawn for interpretation of non-classical elution profiles.

The binding of a ligand on a protein does not change generally its chromatographic retention volume, so that the two forms comigrate and the equilibrium is maintained. However, this condition is not necessarily fulfilled with small acceptors which can enter the gel matrix. A theoretical study has been developed by Cann et al. [44] for these situations. The steady-state binding constant obtained by the method is different from the thermodynamic equilibrium constant. Moreover, when the separation is achieved by capillary zone electrophoresis, the mobility of the complex is often different from those of the constituents. The consequences on electrophoretic profiles and binding constant measurements of the differences in relative mobility values have been described by computer simulation, by Busch et al. [45].

A particular advantage of the Hummel and Dreyer method is the control of the free ligand concentration, on which depends the bound protein ratio, as an independent variable. In other methods, the total ligand concentration is fixed,

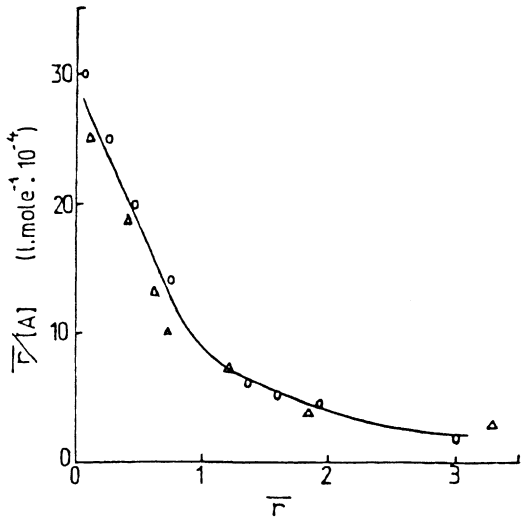

Fig. 2. Scatchard plot for warfarin–HSA binding. ○, Hummel and Dreyer method; △, vacancy peak method. Reprinted from Ref. [6].

the free concentration is measured and contributes to the imprecision of the results.

The parameters of binding are often estimated from the Scatchard transformation ($\bar{r}/A$ versus $\bar{r}$) (Fig. 2). In the most simple case (one class of sites with association constant K), the representation of $\bar{r}/A$ versus $\bar{r}$ is a straight line of negative slope $-1/K$. A graphic method has been proposed to resolve the problem in more complex systems [46]. It must be noted that the non-linear regression programs cannot be applied to the Scatchard transformation, because experimental error is present in the abscissa, instead of being exclusively in the ordinate [47]. Bound versus free ligand concentrations should be curve fitted directly, without transformation, by non-linear least square programs [8].

An internal calibration has been proposed by Hummel and Dreyer: the same amount of macromolecule is injected with increasing amounts of ligand, in the same total volume. By plotting the surface of the ligand peak (at first negative, then positive), over the excess content of ligand (relative to the content in the same volume of eluent), the amount of bound ligand is obtained by zero extrapolation (Fig. 3). We have verified, in the case of the ADP binding on the chloroplast ATPase [41], that this value was independent of the volume injection. This protocol has been simplified by Pinkerton and Koeplinger [8]: the bound ligand concentration was measured by simple subtraction of blank injection (same volume of buffer) from the ligand trough. The vacancy areas obtained from the blank injections with different ligand concentrations provided the external calibration. The two methods have been compared by Sun et al. for the determination of warfarin [7] and tryptophan–albumin [48] binding parameters, the areas being evaluated by different techniques (planimeter, geometry, integrator). They have been found to give reasonably accurate results, the external calibration method being superior for its simplicity and speed. On the other hand, the bound drug amount can be measured

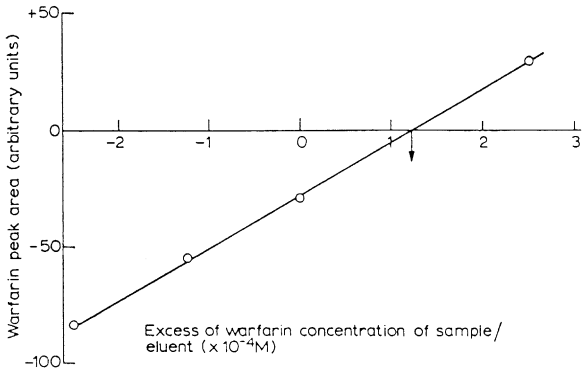

Fig. 3. Internal calibration for binding of warfarin to HSA. Peak area (at 313 nm) as a function of the excess (relative to eluent concentration) of warfarin injected with 3.2×10^{-10} mol of HSA into the column; eluent, 5×10^{-5} *M* warfarin in phosphate buffer (pH 7.4). Reprinted from Ref. [5].

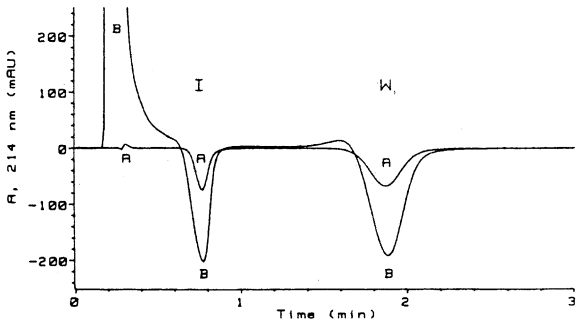

Fig. 4. Hummel and Dreyer chromatographic profile obtained with 5 cm ISRP column from the injection of 50 μl of 54.8 μ*M* HSA (trace B) into a mobile phase containing 40.5 μ*M* warfarin (W) and 60.6 μ*M* buprofen (I) in 0.067 *M* phosphate buffer; flow-rate, 2 ml/min; temperature, 25 °C; trace A is from injection of 50 μl of 0.067 *M* phosphate buffer blank. Reprinted from Ref. [8].

in the positive peak when the detection technique allows to distinguish the ligand (for instance when it is labelled) from the protein: the binding of furosemine [49] or lidocaine [50] to plasma proteins, of dodecylsulfate to albumin [51], or of tropolone to tubulin [52] have been determined in this way.

3. Modifications of the method

3.1. Chromatographic methods

The original Hummel and Dreyer method was developed on soft gel columns and the separation was based on size exclusion. The adaptation to HPLC has greatly improved the resolution and the rapidity and reduced the injection and elution volumes, which is advantageous in the case of expensive products. Moreover, computer-controlled mobile phase delivery systems with low volume syringes have facilitated the use of the method and made it more reproducible [8].

Size exclusion chromatography cannot separate small molecules of similar size and competition between them cannot always be studied in this way, unless they can be distinguished by their spectrum or any label [5]. In order to resolve this problem, other types of columns have been tried.

In internal surface reversed-phase type columns (ISRP) [8], a tripeptide (Gly–Phe–Phe) is covalently coupled, through the N terminus, to glyceropropyl groups bonded on the internal surface of a porous silica packing. On the external surface, phenylalanine is cleaved, using carboxypeptidase A, leaving the hydrophilic glycine. Drugs of low molecular mass are able to enter the pores of the silica and interact with the hydrophobic phase, while large serum proteins are excluded and recovered with high yield, owing to the hydrophilic external surface. The determination of the binding of warfarin on HSA in the presence of a displacer, ibuprofen, has been carried out on this type of column [8] (Fig. 4).

Anion-exchange TSK DEAE 2SW columns have been used to study the simultaneous binding of ADP and ATP on chloroplast ATPase [24]. These two ligands, with identical spectra and similar sizes, are easily separated on anion-exchange columns. Two troughs are visible in Hummel and Dreyer profiles, corresponding to ADP and ATP binding, which can be measured by successive or simultaneous calibration (Fig. 5). On the other hand, the free and complexed protein are held at the head of the column and are not eluted between successive injections. They do

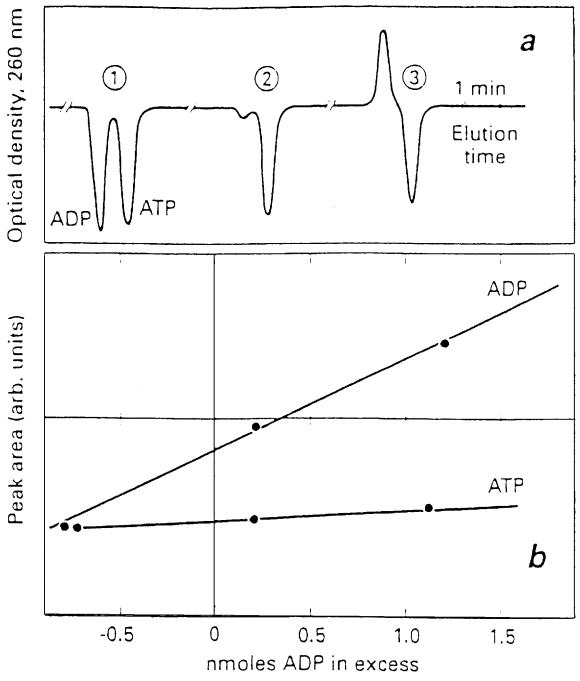

Fig. 5. Chloroplast ATPase–ADP binding measurements in the presence of ATP. (a) Chromatographic profile of ATPase–ADP mixture on an anion-exchange column equilibrated with 1.59×10^{-5} *M* ADP and 1.44×10^{-5} *M* ATP. Conditions: Tris buffer, 75×10^{-3} *M*, pH 8.5; injected ATPase 1.55 nmol, (1) alone, (2) with 1.01 nmol ATP, (3) with 2.02 nmol ADP. (b) ADP peak versus ADP excess. Reprinted from Ref. [41].

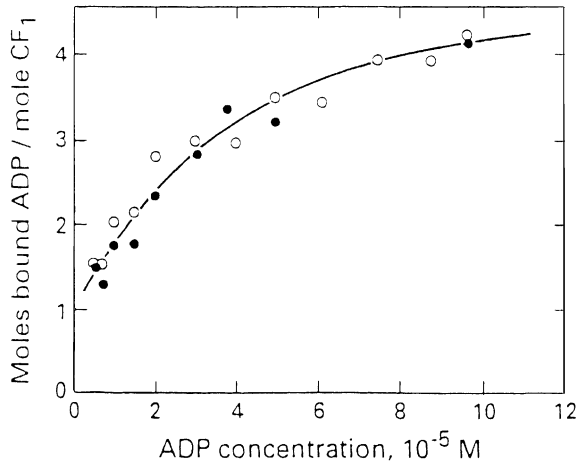

Fig. 6. Comparison of ADP binding on chloroplast ATPase, versus ADP concentration. ●, Measured by gel filtration chromatography; ○, measured by anion-exchange chromatography. Reprinted from Ref. [41].

not intervene in the following binding measurements, since they are in equilibrium with the ligand when a new baseline is established [1]. It has been verified that the binding of ADP alone, measured by the Hummel and Dreyer on a size exclusion column, was identical to that measured on an anion-exchange column, in the same conditions of elution (ionic strength and pH) (Fig. 6). This means that the binding of the constituents on the DEAE groups of the column does not modify the equilibria between them. However, the conditions of the drug binding study (generally, in neutral or slightly alkaline buffers of physiological ionic strength) impose the choice of the anion-exchange column (length, nature and percentage of ionic groups) in order to have a good resolution with acceptable retention times. It may be difficult to achieve this compromise.

In the large zone modification, large volumes of ligand–protein mixtures are injected so that plateaus of concentration are obtained, instead of peaks. This method has been used for the determination of binding of Sr^{2+} on BSA and lactalbumin [21] and of Pb^{2+} on collagen [22], by inductively coupled plasma atomic emission spectrometry (ICP–AES). This modification was also proposed by Brumbaugh and Ackers [42] to avoid eventual modification of ligand binding due to protein dilution during the chromatography.

3.2. Electrophoretic methods

In this technique, the electrophoretic mobility replaces the elution rate (Fig. 7). This method was developed rapidly, as soon as the capillary electrophoresis systems were commercially available, because of its high efficiency, its rapidity and flexibility, the possibility to easily automate and to study simultaneously the binding interactions of different drugs with proteins or mixture of proteins, in practically physiological conditions and at the nanogram level.

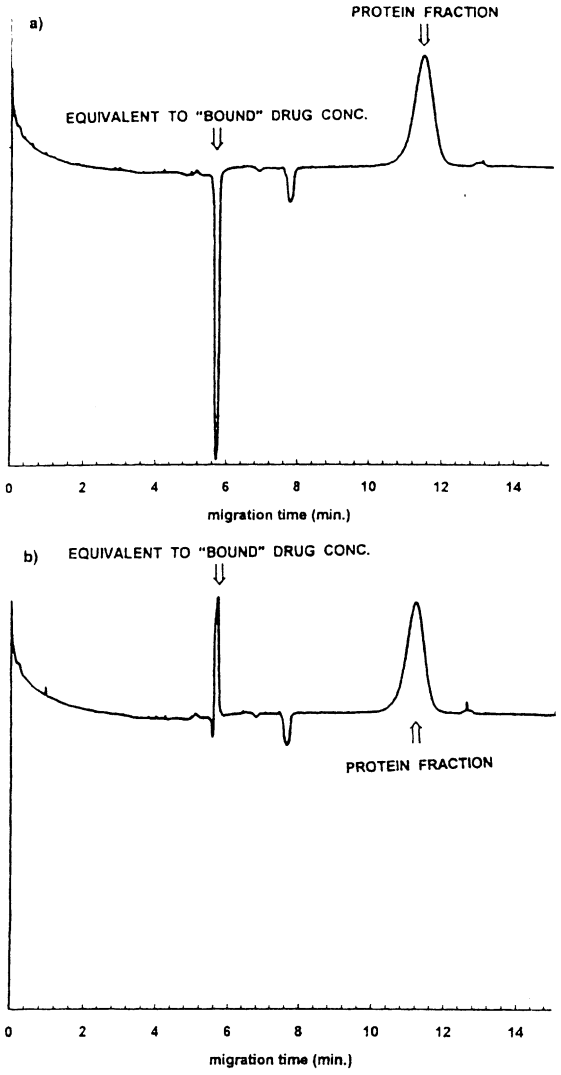

Fig. 7. Representative electropherogram of (*RS*)-carvedilol interaction with human AGP (10 μmol/l). Capillary electrophoresis conditions: concentration of (*RS*)-carvedilol.HCl in running buffer, 20 μmol/l; 75 μm untreated fused-silica capillary (effective length ∼50 cm); hydrodynamic injection (20 s, 20 mbar), applied voltage 20 kV, 25 °C; detection at 210 nm; injected sample: mixed solution of AGP and (*RS*)-carvedilol with drug–protein molar ratio ($D : P$) = 1 (a) and ($D : P$) = 2.5 (b). Reprinted from Ref. [13].

However, some precautions must be taken, such as the control of the temperature inside the capillary, which can be 10 °C higher than outside [53], or the daily flushing and treatment of the capillary, to avoid blockage and to limit protein adsorption on the walls. If irreversible adsorption of HSA may occur, when added to the buffer [53,54], the amount of BSA adsorbed in the case of the Hummel and Dreyer method is small and has a negligible effect on the calculation of the binding parameters [3].

Peak areas rather than peak heights must be measured, because of their deformation, due to slow kinetics and not to adsorption.

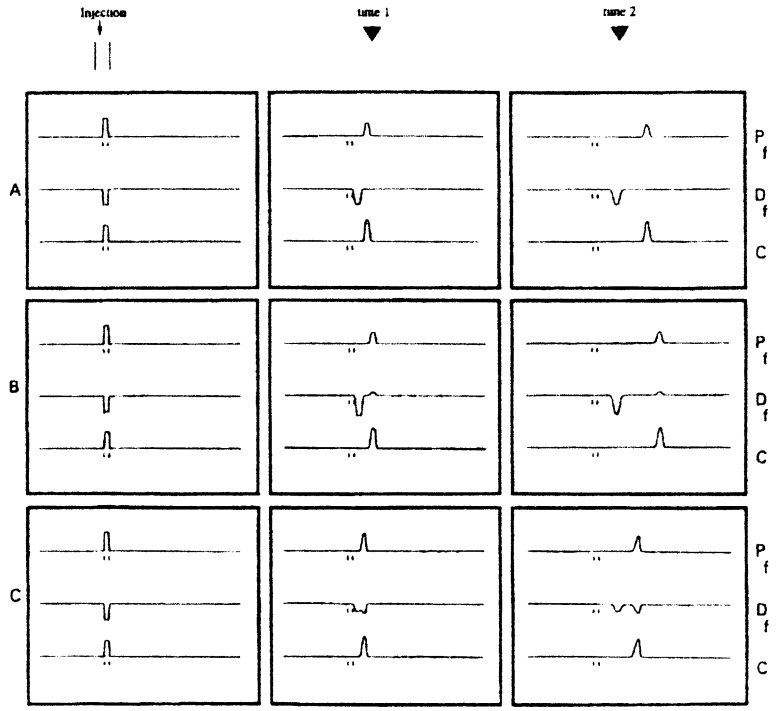

Fig. 8. Simulated concentration-position profiles for the Hummel and Dreyer method, abscissa in position (1 and 2) in capillary. Injection marked by (II). Assumptions: (A) $\mu_C = \mu_P > \mu_D$; (B) $\mu_C > \mu_P > \mu_D$; (C) $\mu_C < \mu_P > \mu_D$. P_f, free protein; D_f, free drug; C, complex. Reprinted from Ref. [45].

Sensitivity of the method was initially rather low, due to the short optical path length of the detector cells. Improvements of the detection limit have been obtained by the use of commercially available high sensitivity optical cells with Z-shaped capillary [55], or by laboratory bubble cell capillary [56].

Contrary to what is generally observed in the chromatographic method, the mobility (μ_C) of the complex C is not always identical to that (μ_P) of the free protein P_f. Computer simulations (Fig. 8, [45]) show that when $\mu_C > \mu_P > \mu_D$ (where μ_D is the ligand mobility), the area of the negative peak is too large and the concentration of free ligand D_f within the migrating zone of P, is higher than in the buffer D_{buff}. This leads to deformation and broadening of that zone, and the average velocity of P becomes a function of its own concentration, via its influence on the D_f value. When D_f and D_{buff} are assumed equal, the calculated binding constants are overestimated. In contrast, when $\mu_C < \mu_P > \mu_D$, the area of the vacancy peak is too low, and a second negative peak is visible, which migrates with the mobility of P. In the migrating zone of P, the concentration of the free ligand is lower than in the buffer. In this case, the binding constants are underestimated. Only when $\mu_C = \mu_P$, the free ligand concentration and the concentration in the buffer are equal and the constants calculated from the multiple equilibria formula are correct.

As in the case of the chromatographic method, competition between protein self-association and ligand binding may lead to an increase of apparent binding constants with dilution. This phenomenon has been shown minimal for warfarin binding when the BSA concentration was kept below 50 μM [9].

Binding of warfarin [3,9] and of buspirone [14] on BSA, of carvedilol [13] on HSA and α_1 acid glycoprotein have been studied by the chromatographic Hummel and Dreyer method and its capillary electrophoresis modification. The two modes of separation have been compared with warfarin on HSA [9] and carvedilol on AGP [13] and gave similar binding constants and number of sites.

4. Comparison with other binding measurement methods

The Hummel and Dreyer method has been compared with other binding measurement methods such as dialysis equilibrium, ultrafiltration, frontal analysis, vacancy peak method, affinity chromatography and the corresponding capillary electrophoresis methods, with regard to their advantages and drawbacks. However, the comparison of the binding parameters is sometimes difficult, because of the differences in experimental conditions.

4.1. Equilibrium dialysis

It was the first method used to study the binding of low molecular mass ligands to macromolecules and it remains still employed because of its easiness and cheapness. A

Table 1
Binding parameters for warfarin–HSA and BSA

Method	n_1	k_1 $\times 10^5$ L/M	n_2	k_2 $\times 10^3$ L/M	$\sum n_i k_i$ $\times 10^5$ L/M	Experimental conditions	Refs.
Hummel and Dreyer chromatography	1.3	2.2	3.8	4.2		HSA, 37 °C	[5]
	1	3.3	2.1	20.3		HSA, 25 °C	[8]
	1	2.5	4	3.9		HSA, 25 °C external calibration	[7]
	1	3.6	4	8.3		BSA	[7]
Hummel and Dreyer electrophoresis	0.7	1	3.2	6.7		BSA, 25 °C 10 kV	[9]
					n_{total}=3.02		
					k_{total}=0.12	BSA, 10 kV (mean values)	[3]
Frontal analysis chromatography	1.2	2.1				HSA, 37 °C	[5]
	1.4	2	3.7	2.2		HSA, 37 °C	[68]
Frontal analysis electrophoresis	1.2	1.5	2.3	5.1		BSA, 25 °C 10 kV (mean values)	[9]
	1.3	1.4	2.6	4.1		BSA, 10 kV (mean values)	[3]
	1	3	2.8	7.4	3.2	BSA, 10 kV	[3]
Vacancy peak electrophoresis	2	0.7	2.7	1.4		BSA,25 °C 10 kV	[9]
	1.9	0.7	2.1	1.5		BSA, 10 kV	[3]
Retention analysis					3.25	HSA, 37 °C	[69]
Frontal affinity chromatography	2.1	0.87				HSA, 4 °C Monomer immobilized	[66]
	2.1	0.82				Dimer immobilized	[66]
Zonal affinity chromatography					2.7	BSA, immobilized	[63]
					3.7		
					4.4	HSA immobilized	[67]
					3.3		
VACE electrophoresis	1	1.3	2.3	1.9		BSA, 10 kV	[3]
Affinity capillary electrophoresis					K_{total} from 0.02 to 0.55	Different concentrations of BSA, 10 kV	[3]
Equilibrium dialysis	1	14	2	18		HSA, 4 °C	[70]
	1	2.3	3.7	6		HSA, 37 °C	[71]
	2	0.9	4	6.7		HSA, 27 °C, 0.1 *M* Tris buffer	[72]
	1	4.7	1	15		HSA, 25 °C	[73]
Ultrafiltration	1.9	0.9				HSA, 37 °C	[74]
Fluorescence titration	1	2.5	2	11		HSA, 22 °C	[75]

semi-permeable membrane separates the free ligand from the mixture of free and bound protein. There is no shift of equilibrium when equal aliquots are taken from both sides of the membrane. The experimental conditions are close to those of the therapeutic assays (high protein content as in plasma). As the constituents are in equilibrium, low affinity binding sites can be theoretically measured, but this possibility is limited by the precision of the difference of ligand concentrations between the two compartments.

The main drawback of the method is the time necessary to reach equilibrium (several hours). The volume of the protein compartment increases during dialysis because of the osmotic effect, so that the dialysis time must be reduced, or high molecular dextran must be added in the free ligand compartment. On the other hand, impurities present in the membrane may bind the ligand, and corrections must be brought to the drug concentrations.

Comparisons have been made with the Hummel and Dreyer method for the binding of ceftriaxone [11], warfarin [7,8], phenobarbital and phenytoin [12] on HSA, of Ca^{2+} on arrestin [20], of steroids (progesterone and 17α progesterone) on cytochrome P450 [35] and of antimitotic agents on tubulin [28], and similar results have been obtained.

On Table 1, the binding constants of warfarin on albumin, given by different methods, are compared: in the case of equilibrium dialysis, the mean values of the numbers of sites and affinity constants are similar to those obtained by the method of Hummel and Dreyer (respectively, $n_1 = 1.25$ and 1.07, $k_1 = 5.5 \times 10^5$ L/M and 2.9×10^5 L/M, $n_2 = 2.7$ and 3.5, $k_2 = 11.4 \times 10^3$ L/M and 9.2×10^3 L/M, $\sum n_i k_i = 5.9 \times 10^5$ L/M and 3.3×10^5 L/M). The slight discrepancies may be ascribed to the differences of temperature, ionic strength or to the nature of the albumins (HSA or BSA).

4.2. Ultrafiltration

Free ligand is separated from free and bound macromolecule by filtration under pressure through a permselective membrane. A large number of samples can be easily and rapidly handled and small amounts of protein are needed. As molecules of solvent and of free ligand cross the membrane at different rates and as this effect is exaggerated at higher pressures, the pressure gradient must be maintained close to zero. In these conditions, the arbitrary restrictions of the volume fraction ultrafiltered are unnecessary and the equilibrium is unaltered, even when the protein concentration increases almost twofold [57]. Practically, the ultrafiltrate volume does not exceed 40% of the initial sample volume, because of the increase of viscosity. Compared to dialysis equilibrium, experimental times are reduced.

The free fractions of phenobarbital and phenytoin in the presence of HSA, determined by ultrafiltration, are comparable to those obtained by the Hummel and Dreyer method [12]. The influence of glycosylation of HSA on the binding of different drugs has been investigated in parallel by the two methods and by frontal analysis [15].

4.3. Zonal chromatography

In certain cases, binding measurements are carried out after separation of the constituents. It is then assumed that the equilibrium is not modified by the separation step, which is not necessarily true, especially when the association constant is not sufficiently high: in the hypothetical case of a complex which obeys first order kinetics during dissociation and associates at the rate of $10^6\ M^{-1}\ s^{-1}$, the loss of binding during separation reaches 10% in 0.1 s for an association constant of $10^6\ M^{-1}$ or in 1 s for $10^7\ M^{-1}$ [58]. Separation techniques must be extremely rapid in the case of loosely bound complexes, which is prejudicial to their efficiency, in particular for zonal chromatography. Thus this method cannot be used with drug protein associations with constants inferior to $10^6\ M^{-1}$ [1].

4.4. Chromatographic and electrophoretic frontal analysis

A mixture of drug and protein is pumped into a size exclusion column or a capillary glass, until saturation. Because of differential migration, plateaus form as the large sample reaches the outlet. The free ligand is measured from the last eluting plateau. When the retention rates or the mobilities of the free protein and that of the complex are identical (which is generally the case in size exclusion chromatography), all the species are in equilibrium, and the height of the plateau of the eluting free drug reflects the correct concentration in the injected sample [45]. If this condition is not fulfilled, and as for the Hummel and Dreyer method, the concentration of the free drug is not constant in the protein migrating zone and the binding parameters are over- or under-estimated. A constant concentration of protein is advantageous when the drug solubility is low or when the extent of binding depends on protein association. However, large amounts of protein and ligand are used, which is expensive and reduces the lifetime of the columns. Moreover, tailing makes difficult the determination of the free drug plateau. Irreversible adsorption of HSA used as a running buffer additive occurs on a glass wall in capillary electrophoresis [13]. On the other hand, in capillary electrophoresis, there is no systematic influence of the separation voltage [3].

The binding parameters of the warfarin–HSA primary site measured by the chromatographic frontal analysis are in agreement with those of the Hummel and Dreyer method, under similar conditions, but there are discrepancies concerning the second site [5,8]. Comparisons between these two methods have been carried out for the calmodulin–tubulin association [26] and, as mentioned above, for the study of drug affinity of glycosylated HSA [15]. In capillary zone electrophoresis, frontal analysis curves obtained with warfarin on BSA are reproducible and the binding constants fit well with those of the Hummel and Dreyer method: mean values of $n_1 = 1.2, k_1 = 2 \times 10^5$ L/M, $n_2 = 2.6, k_2 = 5.5 \times 10^3$ L/M, $\sum n_i k_i = 2.4 \times 10^5$ L/M (instead of, respectively, 1.07, 2.9×10^5 L/M, 3.5, 9.2×10^3 L/M, 3.3×10^5 L/M, see above).

4.5. Chromatographic and capillary electrophoresis vacancy peak methods, vacancy affinity capillary electrophoresis (VACE)

Sebille et al. [1,6] have proposed the vacancy peak method (called equilibrium saturation method), in which the macromolecule and the ligand are in constant equilibrium in the eluent on a size exclusion column. A small volume of buffer is injected, which produces two troughs: the first negative peak expresses the deficit of protein and complex (which have generally the same retention rate in size exclusion chromatography), the second one is that of free ligand. One of the components is varied, the other is kept constant. The mean number of molecules of bound ligand and the binding parameters can be obtained both from the area of the second peak and from its retention volume. The two ways have been shown to be equivalent. The bound ligand ratio remains constant during chromatography, even when it depends on protein association. Here too, the constant concentration of protein allows the use of low solubility drugs, which is an advantage over the Hummel and Dreyer method. The possibility to study the competitive binding of ligands at the same site, if the column can separate the different constituents, is another advantage of the method. However, it requires large amounts of protein and drug.

Warfarin binding on HSA has been measured by Sebille et al. and compared to that given by the Hummel and Dreyer method [6]. The same comparison has been performed by the electrophoretic technique by Busch et al. [3] with BSA. n_1 values have been found significantly higher (mean

value 1.95) and affinity constants lower than with the other methods.

In the vacancy affinity capillary method (VACE), the shifts in the migration times are monitored, instead of the peak areas [3,45,59]. The ligand concentration is variable in the normal VACE, while the protein is variable in the reversed mode. The plot of the mobility of the drug in the presence of protein, versus the total drug concentration contains both information on the absolute number of binding sites of *P* and on their association constants, while mobility of the protein in the presence of drug enables to obtain only binding constant values [59]. However, computer simulations show that, even when the mobilities of the free protein and of the complex are identical, the area of the drug vacancy peak in the migrating zone of *P* does not reflect the free drug concentration in the buffer, because drug is also generated by dissociation of the complex [45]. As a result, the values found for the binding constants are too high. The error depends on the differences in the ligand, protein and complex mobilities and can be minimized by small buffer injections [45]. The free drug concentration can be calculated from the total concentration and from binding parameters, estimated in an iteration step, and the calculated mobilities are compared to the observed values. With multiple equilibria, assumptions must be made, concerning the change in mobility (for instance, in proportion to the number of ligands). Comparison of binding parameters measured by this method and by that of Hummel and Dreyer has been given by Busch et al. [3]: in the case of the binding of warfarin on albumin, the numbers of sites are similar, but the binding constants are smaller.

4.6. Retention analysis, affinity chromatography and affinity capillary electrophoresis (ACE)

Contrary to the Hummel and Dreyer method, in chromatographic retention analysis, the concentration of macromolecule is maintained constant in the eluent, and a small amount of ligand is injected. The retention volume of the drug (positive peak) is a function of its concentration, of the concentration of protein, and of the affinity constants. The total affinity $\sum n_i K_i$ is obtained from the difference between the drug retention volume in the absence of protein and the limit of this volume when the concentration of the drug tends to zero, at known protein concentration. It must be verified that this limit exists for low enough injected drug amounts [1]. This method, originally described by Uekama et al. [60], was developed by Fujimura et al. [61]. The value of warfarin–albumin binding constant obtained by this way is similar to that of Hummel and Dreyer ($\sum n_i k_i = 3.25 \times 10^5$ L/M, compared to 3.32×10^5 L/M, Table 1). The same result has been found with the model steroid—cyclodextrin [31]. Here too, large amounts of macromolecule are required.

In the affinity chromatography, the macromolecule is immobilized on the support and the ligand binding is studied by zonal or frontal elution. Equations have been given for evaluating association constants [62,63]. The binding constants of different drugs on BSA [64] or HSA [62] (reviewed in Ref. [1]) or on β-cyclodextrin polymer [32] have been found similar to those obtained by other methods, including the Hummel and Dreyer method: mean value of $\sum n_i k_i$ for the warfarin–albumin model = 3.52×10^5 L/M, compared to 3.32×10^5 L/M, Table 1. However, the properties of immobilized proteins may be different from those in solution. The affinity interaction of immobilized HSA for phenylbutazone is somewhat larger than that measured in solution [62]. On the other hand, Nakano et al. have shown that, in the case of BSA linked to agarose through a six-carbon spacer [64], the binding properties are identical to those of the soluble protein. With this method, the determination of the association constants of different drugs and their competition is possible with reduced consumption of proteins, when the same column can be used.

The affinity capillary electrophoresis (ACE) method relates the changes of electrophoretic mobility of the injected component to the concentration of the other component in the buffer. The experimental conditions are either identical to those of the Hummel and Dreyer method or to those of retention analysis, but the mobility of each free constituent is assumed to be different from that of the complex. Only binding constants can be determined by this method, the number of binding sites has to be determined from other methods of measurement. Here also, in the case of successive bindings, assumptions must be made concerning the change of mobility with increasing complexation (in proportion to the number of ligands, for instance). Another drawback is the fact that, in the migrating zone of the injected component, the free concentration of the other component cannot be directly measured. The buffer concentration is usually taken as an approximation, which introduces a systematic error. It can be minimized by decreasing the injected amount. Comparison of ACE with Hummel and Dreyer and other binding measurement methods shows similar data [3,65].

4.7. Physical methods

Besides these methods, physical techniques, based on the physical effects of the binding (calorimetry) or on the modifications of the properties of the constituents (fluorescence emission, UV, IR absorption spectroscopy, circular dichroism, NMR) have been used to determine the binding constants. These methods are indirect and involve assumptions which are not always checked. Comparisons with the chromatographic or electrophoretic methods are rare. Association constants of different drugs with β-cyclodextrins [32] determined by the Hummel and Dreyer method have been compared to values obtained by physical methods and the binding of nucleotides on chloroplast H^+ ATPase by various physical techniques has been reviewed [41]. In the case of the warfarin–albumin model, there is a good agreement between the fluorescence titration and the Hummel and Dreyer method: $n_1 = 1, k_1 = 2.5 \times 10^5$ L/M, $n_2 = 2, k_2 =$

11×10^3 L/M, compared to, respectively, 1.07, 2.9×10^5 L/M, 3.5, 9.2×10^3 L/M (Table 1).

5. Conclusion

The example of the warfarin–HSA or BSA binding shows that the Hummel and Dreyer method gives similar values to those of other classical methods, in the same experimental conditions. The choice may be guided by the material availability and the practical constraints of each circumstance.

This is a direct method of measurement of ligand binding, which is less exposed to artefacts than indirect physical methods and its main advantage is the control of the free ligand concentration as an independent variable, on which depends the bound site ratio.

However, some critical factors may affect the accuracy and the reproducibility of the results and must be carefully controlled: the temperature of the mobile phase, in chromatography [31], or inside the capillary, in electrophoresis [13] (a decrease in temperature inducing an increase of the binding constants) [8], the ionic strength of the buffer, when ion-exchange columns are used [24,41], or the sample diluent organic content in reversed-phase chromatography [31]. In certain cases, self-association of proteins may have an influence on ligand–protein interaction, a decrease of the binding constants being observed when the protein concentration increases [3,42]. In capillary electrophoresis, correct results for the binding parameters can only be obtained when the mobilities of the protein and the complex are equal [45].

The progress in HPLC technology and in automation have reduced the sizes of columns and the volumes of eluent, which is advantageous in the case of expensive drugs or macromolecules. The use of reversed-phase and anion-exchange columns would extend the possibility of separation and allow to measure simultaneously the affinity of different ligands for the same receptor, and their competition. Because of its resolution power in quasi physiological conditions, the Hummel and Dreyer method in capillary zone electrophoresis will be certainly increasingly used for binding measurements.

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