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Review

Separation procedures used to reveal and follow drug–protein binding

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1 INTRODUCTION

General biological processes involving the interaction of a biopolymer, *i.e.* a macromolecule, with a small molecule may be found in enzymology, immunology, pharmacology, biology and more generally in medical sciences. More specifically, in pharmacology, the small molecules are usually drugs and the macromolecules are proteins. These macromolecules act as receptors and thus generate a pharmacological effect via a stimulation mechanism. They may act also as acceptors, "silent" proteins, which may either transport drugs from blood to tissues or store them in the body. The enzymes will bind the drugs and transform them into metabolites. A common step for all these biochemical effects (transport, storage and metabolism) is the binding of a drug to a specific protein. Therefore interactions between ligand and macromolecules are obviously quantitatively important as well as qualitatively significant.

The methods for investigating these interactions may roughly be divided in two groups: spectroscopic techniques and non-spectroscopic ones. The latter involve separation of the free ligands from bound species. They include soft-gel chromatography, high-performance liquid chromatography (HPLC), ultrafiltration, ultracentrifugation and equilibrium dialysis. The spectroscopic methods are briefly mentioned here and include ultraviolet, visible and fluorescence spectroscopy, nuclear magnetic resonance, electron spin resonance, optical rotatory dispersion and circular dichroism.

Separation methods allow the determination of the ligand concentration (mainly the free ligand), whereas the spectroscopic techniques are able to characterize the ligand–macromolecule complex (*i.e.* the bound ligand). The results obtained from these different methods may be considered as complementary rather than competitive.

In most cases, the drug–protein interactions are analysed according to the Scatchard model [1], assuming that the ligand is bound to m classes of identical, independent binding sites. From the various chemical equilibria involved, the fraction $\bar{r}$ of bound ligand molecules per protein molecule is given by:

$$\bar{r} = \frac{[B]}{[P]} = \sum_{i=1}^{m} n_i \frac{k_i[F]}{1 + k_i[F]} \tag{1}$$

where [F], [B] and [P] are, respectively, the concentrations of free drug, bound drug and protein; n_i is the number of sites of class i and k_i is the corresponding association constant. The drug–protein data analysis generally assumes two types of binding site on the protein, and the binding parameters characterizing the interaction are then n_1, k_1, n_2, k_2. The methods involving species at equilibrium give directly the equilibrium isotherm, *i.e.* the ratio $\bar{r}$ as a function of free ligand concentration, and an appropriate mathematical treatment leads to the binding parameters. This is the general case for all the methods used in the study of

drug–protein interactions, except those using zonal elution chromatography and retention data analysis. By extrapolation to zero amount of solute injected, zonal elution at high dilution permits the determination of a global binding constant K':

$$K' = \sum_{i=1}^{m} n_i k_i \tag{2}$$

The choice of a particular method depends on the stability of the ligand–protein complex and on the accuracy needed, *i.e.* the bound ligand percentage, the binding parameters or the molecular recognition ability of the protein.

At present, three separation methods are widely used: equilibrium dialysis, ultrafiltration and HPLC.

2. EQUILIBRIUM DIALYSIS

Davis [2] and Klotz [3] were the first to use equilibrium dialysis to study the binding of low-molecular-mass ligands (MW $<$ 1000) to macromolecules. This method is still widely employed to determine the binding parameters characterizing the interaction of ligands or drugs with biopolymers such as plasma proteins [4–7].

Equilibrium dialysis is theoretically the most accurate way to determine free and bound ligands because the equilibrium is not shifted when aliquots are taken from both sides of the dialysis membrane. Special attention was paid to reduce the time for reaching the diffusion equilibrium and to improve the measurement reproducibility with standard protocols fixing the experimental conditions [8].

In a typical equilibrium dialysis experiment the macromolecular solution is separated by a semi-permeable membrane from the ligand solution. If there is no alteration of ligand or protein(s), the system reaches an equilibrium state known as "steady state". Since, at steady state, the free ligand concentrations are equal on both sides of the dialysis membrane, the concentration detected in the compartment containing the protein(s) is due to the sum of bound and free drug concentrations, whereas the concentration detected in the other side is that of the free ligand only. Therefore, the concentration of bound ligand is equal to the difference between the total ligand concentrations (bound plus free) of the solutions present in each compartment (Fig. 1).

According to Fick's law the ligand diffusion rate is a function of the membrane surface area and thickness, of the concentration gradient, of the compartment volumes and of the diffusion coefficient. This last quantity, which characterizes a particular ligand molecule, is a function of the ligand molecular mass and the temperature (the dialysis rate increases with temperature). Obviously the membrane should be chosen so as to obtain the largest ligand diffusion rate together with the highest retention capacity for all the protein(s).

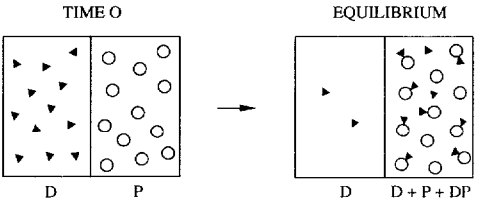

Fig 1 Schematic representation of equilibrium dialysis. At equilibrium, the concentration of drug–protein (○◀) interaction (DP) is obtained from difference between the protein compartment (D) representing the free drug (▲) concentration The free protein (○) concentration is P.

Equilibrium dialysis systems (Dianorm®, Munich, F.R.G.) with a given ratio of the membrane surface to the cell available volume, have been specially designed [9] to meet the optimal requirements of binding studies. The higher this ratio, the shorter the dialysis time. Recently a simple and inexpensive system has been developed, using 1.5-ml Eppendorf® microtubes, which may be discarded after use [10].

The membrane must be carefully chosen and several features have to be considered. The membrane thickness is an important factor because the rate of dialysis is roughly proportional to its reciprocal Moreover, the non-specific binding of a ligand to a membrane increases with membrane thickness. The presence of ionized sites on the membrane surface may also be a drawback for drug–protein binding studies, since additional interactions will take place.

Nitrocellulose commercial membranes are not suitable because of their high adsorption activity towards many compounds and their relatively large thickness. However, natural cellulose membranes (Visking®, Diachema®, Spectrapor®) greatly reduce these effects and enable the complete retention of the protein(s), with high dialysis rates and relatively weak adsorption of the reactants. It is now possible to find membranes with pore dimensions suitable for various experimental needs.

However, commercial membranes, even the most expensive ones, may contain adherent impurities that must be removed by washing with distilled water or 0 01 *M* acetic acid (hot or cold) and then by soaking in the experimental buffer. Finally, the membrane molecular mass cut-off will be selected according to the protein studied, but it must be as large as possible for a fast diffusion of the ligand.

Some years ago, Kurz *et al.* [11] compared different methods for binding measurements (equilibrium dialysis, ultrafiltration, ultracentrifugation, gel filtration) and concluded that the values determined from equilibrium dialysis measurements give a good estimation of the ligand binding ratio. Equilibrium dialysis has the advantage of being easy and cheap. Moreover, the equilibrium between free and bound drugs is completed with no variation of protein concentration during the experiments. When consecutive binding determinations are carried out, the equilibrium is not disturbed when equal sample volumes are simultaneously re-

moved from both sides of the membrane. This is not the situation with ultrafiltration methods. An additional advantage of equilibrium dialysis is its ability to allow measurements with low-affinity binding sites. In the case of ultrafiltration technique, with drug–plasma protein association, the ligand–protein complex could be almost dissociated during the time required for the separation. However, a disadvantage of equilibrium dialysis is the fact that high concentrations of protein binding sites are required for measuring bound ligand fractions over 20% when dissociation constants are above 10^{-5} *M*.

The drawbacks of equilibrium dialysis are mainly technical. Often a significant amount of ligand may be adsorbed on the membrane and cell walls, thus leading to a decreased concentration of the available ligand. This non-specific binding may be quantified after dialysis, but sometimes it may be as large as 50% [11]. For calculating free ligand amounts, one should not use the initial total ligand concentration, but the ligand concentration found in the protein compartment when the equilibrium is achieved. Therefore, the ligand amount adsorbed by the dialysis devices and membranes must be checked and the non-specific binding eventually taken into account for further calculations [12]. In order to dissolve a ligand, such as a drug, small percentages of organic solvents added to the solution are required, but it has been shown that their presence may change the ligand binding equilibrium. For instance, additions of 1–5% ethanol decreases the binding ratio by *ca.* 12% [13].

At high concentrations of protein(s), for *e.g.* plasma proteins, volume variations caused by osmotic equilibration are observed, which may result in an apparent protein dilution effect. The volume shift will increase with dialysis time [14,15]. Especially with strongly bound ligands, this will lead to an overvaluation of the free ligand concentration, which may reach 60% [16]. Several methods have been introduced to reduce or solve this problem. If possible, experiments have to be carried out with low protein concentrations, or by adding polymers, such as high-molecular-mass dextran (MW 70 000), to the ligand-free compartment [17,18]. One may also reduce dialysis time (less than 3 h) in order to lower oncotic pressure effects [14], or add the ligand to the protein compartment [19,20], or use competitive equilibrium dialysis [21]. Tozer *et al.* [15] have developed a mathematical model to give a correction for volume shifts in the protein compartment leading to a decrease of the concentrations of protein and ligand.

The free and bound drug concentrations in a plasma containing a drug can be estimated by equilibrium dialysis provided that the diffusion of the free drug into the buffer side is taken into account. In order to have an accurate value of the initial bound drug concentration or its free fraction in plasma, the results obtained from equilibrium dialysis must be corrected using a dilution factor [22]: dialysing a plasma containing a drug against the same volume of buffer leads to a change in the volume occupied by the drug or the protein(s). It can be concluded that a drug, the binding of which to a protein is not saturating in the range of therapeutic concentrations, shows a constant free drug fraction (fu), but

its concentration as a free drug is lower than it was in plasma before dialysis. Conversely, a drug, the binding of which to a protein is saturating in the range of therapeutic concentrations, shows a variable free drug fraction (fu), but its concentration as a free drug remains roughly constant as the dialysate volume increases.

With high-molecular-mass ligands, the time needed to reach equilibrium may be longer than 12 h because of steric hindrance. The dialysis technique has then to be modified in order to permit ligand binding determinations over shorter times. The technique is known as kinetic dialysis [23]. In the dialysis cell the dialysis membrane separates the protein compartment from another one in which a buffer solution flows at constant rate. The ligand added to the protein(s) will pass through the membrane to the buffer side at a rate proportional to the free ligand concentration in the protein side. The calibration of the ligand diffusion rate with and without the protein(s) will give the values of free and bound ligand concentration. The steady state will be reached in less than 1 min with small ligands when using specially prepared dialysis membranes. Moreover, kinetic dialysis allows the use of the same protein concentration sample for several binding measurements at different ligand concentrations.

In order to reduce the experimental time in equilibrium dialysis, Hwang and Bayne [24] proposed another approach known as dynamic dialysis. The ligand is added into the protein side instead of being added into the buffer compartment. In such a system, less time is needed for the free ligand concentration to reach its equilibrium value: the higher the binding, the shorter the time needed to reach a steady state. This approach can be performed with kinetic effects too, when the ligand concentration in the buffer side can be measured at any time, provided that the ligand binding is linear over the range of concentrations studied.

It is also difficult to study accurately the binding of ligands that are poorly soluble in aqueous solution, because they may aggregate and adhere to the membrane surface. Recently, this problem was solved for palmitate by measuring the transfer rate of radiolabelled palmitate through a membrane separating two solutions with the same human serum albumin (HSA) concentration [25]. At time intervals long enough for the labelled ligand to equilibrate, the radioactivity is measured in both compartments of the dialysis cell.

One should note that equilibrium dialysis is the most accurate technique for studying drug–plasma interactions because, in therapeutic conditions, the protein concentrations are often much larger than the total drug concentration. The bound drug concentration is then larger than its free concentration (high binding percentage). On the other hand, for low protein concentrations or for total drug concentrations larger than the protein saturation, the concentration of bound drug is slightly higher than that of free drug in both compartments. This latter limitation is perhaps the most significant disadvantage of studying ligand–receptor interactions by equilibrium dialysis.

3 ULTRAFILTRATION

This method of separation of the bound ligand from the free one is extremely popular because of the easy handling of a large number of samples, as well as the commercial availability of a variety of filtration devices (Amicon®, Sartorius®). In the ultrafiltration of a protein solution containing a ligand, the pressure difference forces the buffer containing free ligand through a permselective membrane. The total volume of ultrafiltrate plus retentate solutions equals the initial solution volume. Moreover, the free ligand concentration will be constant in the ultrafiltrate and the retentate [22].

Any decrease in the concentration of free ligand would result in bound ligand dissociation according to the law of mass action. In ideal ultrafiltration, the free ligand volume remains constant, and the concentrations of bound ligand and free protein sites increase at the same rate as the total protein concentration. The bound ligand to free protein concentration ratio in the retentate is then independent of the ultrafiltrate volume [22–26]. Nevertheless, it has been shown that the free ligand concentration is constant during the process, provided that the ultrafiltrate volume does not exceed 40% of the initial volume introduced [26].

This technique is mainly used to determine the free ligand fraction and sometimes the protein–ligand binding parameters [27,28], using a micropartition system (Amicon®), based on a driving force for ultrafiltrates produced by centrifugation at 10 000–20 000 m s^{-2}.

Compared with equilibrium dialysis, ultrafiltration is faster and may be used with unstable protein samples. Moreover, because of the small volumes of ultrafiltrate needed, the method allows the use of small amounts of protein. At high protein concentrations, the problem of oncotic pressure found in equilibrium dialysis is overcome with the ultrafiltration technique.

The main drawbacks of the ultrafiltration technique are similar to those of equilibrium dialysis. Filtration membranes, the composition of which differs from those used in dialysis experiments, may bind many lipid-soluble ligands and thus give an undervaluation of the free ligand concentration in the ultrafiltrate. Nevertheless, this non-specific binding is sometimes close to that observed with cellulose dialysis membranes [29]. For instance, the loss of propranolol during ultrafiltration experiments is considered to be due to additional binding to the membrane and to the O-rings [30]. To overcome this problem, one may either saturate the ultrafiltration membrane with the unlabelled ligand (when radioactivity measurements are used), or coat it with a siliconizing solution.

The undervaluation of the free drug concentration in the ultrafiltrate may also be due to the retention of high-molecular-mass ligands [11], such as suramine (MW = 1297). Not all the filtration membrane pores have the same diameter, and ligands will pass through the membrane at different rates, while water diffusion is easier. Thus, compared with the ligand concentration in the initial solution, the ligand is diluted in the ultrafiltrate. This phenomenon, known as the

"sieve effect", is more pronounced at higher filtration rates and larger molecular masses. So membrane pores must be large enough to reduce the "sieve effect" caused by the easier passage of water than of free ligand. Proteins may also lead to the same effect since a protein film coating on the membrane behaves like a second membrane (concentration polarization).

For all these reasons, it is better to discard the initial ultrafiltrate (*ca.* 5% of initial volume) in order to avoid the higher free ligand concentration observed at the beginning of the filtration, when the ultrafiltrate volume increases. In practice, there is no "standard" method for protein-binding measurements. Most investigations use equilibrium dialysis because the main advantage of the method is to keep free and bound ligands in solution with no separation of the species and therefore no alteration of the chemical equilibrium. This method may still be considered as a reference for calculating the binding parameters of a ligand to a given protein However, ultrafiltration seems to be a better choice for the evaluation, for clinical purposes, of the bound drug fraction, since dilution effects are reduced and experimental times are shorter than with equilibrium dialysis [31]. Kurz *et al.* [11] have developed a sequential method in which the sample is first dialysed to equilibrium and then the retentate in the protein compartment is ultrafiltered. The free drug fraction is calculated as the ratio of the ultrafiltrate concentration to that of the dialysis retentate.

4 LIQUID CHROMATOGRAPHY WITH DRUG AND PROTEIN IN MOBILE PHASE

Chromatography is the most popular separation method in biochemistry and it was applied very early to obtain the parameters characterizing the binding of a drug to a protein. Since the review written by Wood and Cooper [32], dealing mainly with the use of soft gels such as Sephadex®, the development of high-performance equipments has extended the scope of chromatography to numerous binding parameter measurements [33]. Two kinds of experimental approach can be considered: either all the species at equilibrium are dissolved in the mobile phase or one of them is immobilized, as a stationary phase, on the chromatographic support. The latter is the well known affinity chromatographic technique. When none of the species to be studied is immobilized, different methods are available. The choice must take into account various parameters, such as the binding capacity, the species solubility, the available amount of ligand, etc.

4.1. Quantitation of the drug–protein complex by zonal elution

A small sample volume containing a mixture of the drug and the protein is injected at the column inlet and is eluted by an aqueous buffer solution. A size-exclusion mechanism or an ion-exchange one, for example, separates the complex from excess ligand. Generally the complex elutes first from the column and is followed by a second peak corresponding to the free ligand (Fig. 2B) Quantita-

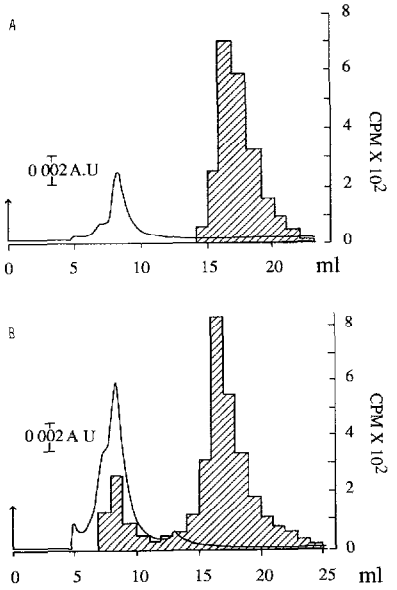

Fig 2 Chromatograms showing the elution of prednisolone incubated with albumin (A) and with serum (B) The histogram represents the radioactivity (Reproduced with permission from ref 35)

tive measurements of the complex and free ligand concentrations allow the calculation of the binding parameters.

The validity of the results depends on the interaction kinetics, since the complex may be dissociated by the dilution resulting from the elution transfer as well as by the drug retention on the stationary phase. The theoretical aspects of this process have been analysed [34], and the conditions of complex stability that allow the method to be used have been calculated. When the association constant is over $10^7\ M^{-1}$, the dissociation rate is small enough to keep the ligand–protein complex intact during the chromatographic elution transfer. The results of Loo *et al.* [35] illustrate this point since they show the presence of the corticoid-binding globulin (CBG) in human plasma and measure the affinity of prednisolone for this protein and albumin. These experiments demonstrate the stability of the CBG–prednisolone complex ($K_{ass} = 3 \cdot 10^7\ M^{-1}$) and the fact that the albumin–prednisolone complex ($K_{ass} = 3 \cdot 10^3\ M^{-1}$) is completely dissociated during elution (Fig. 2A).

More generally, the quantitative analysis of peak areas in zonal chromatography may be used when the eluted sample is moderately diluted and the protein–ligand dissociation kinetics are slower than the chromatographic elution process. As many examples concern drug–protein binding associations with constants under $10^6\ M^{-1}$, other chromatographic methods have been developed that do not alter the complex stability.

4.2. The Hummel and Dreyer method

The chromatographic method most widely used for measuring drug–protein interactions was first described by Hummel and Dreyer [36] in 1962. The technique requires a column that is able to separate the drug and the protein by a size-exclusion mechanism. At first, soft-gel columns were used, but gradually the use of rigid supports and HPLC equipment allowed the development of the method by reducing the analysis time. Ion-exchange columns have recently been successfully introduced [37] and applied to binding studies

The technique uses an eluent containing the drug to be studied dissolved at a given concentration. A small amount of protein is injected into the column, and a typical elution profile is shown in Fig. 3. A positive peak appears corresponding to the ligand–protein complex, and a negative one emerges at the drug retention volume. The negative peak (or trough) area depends directly on the amount of bound drug. The number of bound drug molecules per macromolecule is fixed by the drug concentration in the eluent and therefore is constant during elution.

The bound drug may be quantified from either internal or external calibration. Hummel and Dreyer [36] first described the internal calibration method. When increasing drug concentrations are injected with the protein the trough area will decrease, and for large enough concentrations a positive peak may even appear. A simple interpolation permits the drug concentration for which the trough vanishes to be determined. This value allows the calculation of the bound drug amount. Using HPLC, we have applied this method to the determination of the

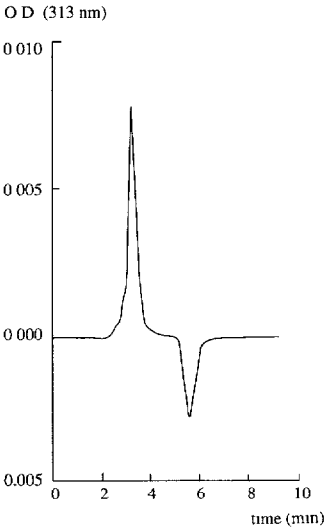

Fig 3. The Hummel and Dreyer method Chromatographic signal obtained by injecting HSA in an eluent containing warfarin Sample, 12.5 μl of HSA (2 g l^{-1}), eluent, warfarin 0 5 10^{-6} *M* in 0 067 *M* phosphate buffer (pH 7 4); flow-rate, 0.5 ml min^{-1} (Reproduced with permission from ref 38)

warfarin–HSA binding parameters [38]. The external calibration method compares the trough area with those obtained from the usual calibration techniques in which increasing amounts of drug are injected in the aqueous buffer. Sun *et al.* [39] have shown that the two calibration methods give similar results when applied to the warfarin–albumin interaction.

Another possible approach for drug-binding studies is the evaluation of the drug amount included in the positive peak. This method is very useful when the drug detection technique is distinct from that of the protein. For example, the binding of a labelled ligand, such as labelled dodecylsulphate, to HSA was studied by Allen [40]. The same method was chosen by Andreu and Timasheff [41] to measure the tropolone methyl ester–tubulin binding. Recently, evidence of the binding of labelled lidocaine to several plasma proteins was given by Abe *et al.* [42], who measured the ligand concentration included in the size-exclusion HPLC protein peak. This approach was previously described by Forrey *et al.* [43], who used labelled furosemide to study the binding of this drug to plasma proteins.

A disadvantage of the Hummel and Dreyer method is the decrease of protein concentrations observed during the elution process. In contrast to the multiple equilibria theory [44], which establishes that the $\bar{r}$ ratio is independent of protein concentrations, experiments [45] have shown that the association constants decrease with the protein concentration. The influence of the self-association of proteins on the ligand–protein interaction may explain this phenomenon. The problem was overcome by Brumbaugh and Ackers [47], who injected large sample volumes into the column in order to obtain plateaus of protein concentration instead of peaks. Another approach consists of using the saturation method described in next section.

A good resolution between the protein peak and the negative one is the main requirement of the Hummel and Dreyer method, and erroneous measurements may result from tailing protein peaks. This tailing may have its origin in slow kinetic processes or may occur from self-association induced by ligand interactions. Cann and Hinman [48] and Steiner [49] have analysed this last point from a theoretical point of view.

In principle, drug adsorption on the chromatographic gel does not interfere with protein-binding measurements. When the column is saturated with an eluent containing the drug, the drug concentration in the mobile phase is actually the free ligand concentration, which is in equilibrium with the protein. Nevertheless Zaton *et al.* [50], using soft gels for measuring the benzylthiouracil–HSA binding, have obtained results that differ significantly from dialysis measurements A better agreement was obtained when the free drug concentration was calculated by subtracting the amount of drug adsorbed by the gel from the amount in the mobile phase. However, this correction is not supported by a valid theoretical background.

The Hummel and Dreyer method is largely used with soft gels such as Sephadex®. Examples include the binding of sulphonamide [51], peptides [52] and drugs

such as warfarin and phenylbutazone [53]. Recently the use of rigid gels such as TSK® (Toyo Soda) or LiChrosorb® diol (Merck) has reduced the analysis time from hours to few minutes. Moreover, the improved resolution due to the use of HPLC equipment gives more accurate measurements, and drug labelling is no longer required. We used this method to study the binding of warfarin, furosemide [38] and diazepam [54] to HSA. The same technique was also applied to determine the binding parameters of propranolol to α_1-glycoprotein acid [55] and other plasma proteins [56]. As already mentioned, a complete study of the calibration method was described by Sun and co-workers and applied to the study of the warfarin–HSA binding [39] and the tryptophan–HSA binding [57]. A TSK SW® (Toyo Soda) column was used by Williams *et al.* [58] to determine the colchicine–tubulin binding parameters. They have shown that the high pressures in HPLC experiments, which might induce a depolymerization of the microtubules, do not affect measurements of tubulin binding because this phenomenon is too limited to disturb the association equilibrium.

The Hummel and Dreyer method is useful for studying the competitive interactions of two drugs towards the same protein. This approach was first described by Fairclough and Fruton [52] with soft gels, and the method was then transferred to rigid supports to study the competition of warfarin and furosemide for the same HSA binding site [38]. Recently, ion-exchange supports were used to measure the simultaneous binding of ADP and ATP to the spinach coupling factor F1 [37]. This work proves the validity of using the Hummel and Dreyer method with a system based on an ion-exchange separation mechanism and leads to new insights into binding measurements. Until now the method has been limited to drugs that are weakly retained in aqueous media on size-exclusion supports. With these supports, however, some problems may arise because the solvent conditions needed for binding studies, generally an aqueous buffer at pH 7.4, determine the nature of the eluent. The retention volume may then be too small (poor separation from the protein) or too large (strong or irreversible retention). The use of ion exchangers may resolve this problems and give better peak resolution Moreover, the separation of the different fractions of a complex protein mixture enables the positive peaks to be measured and makes possible the targeting of individual fractions by a drug.

4.3. Frontal elution

In frontal chromatography, a solution is applied continuously to the column until the column is equilibrated. The elution profile at the column outlet reaches a plateau equal to the input concentration. The use of frontal analysis to study associations between two different species was first introduced by Nichol and Winzor [59] for protein association determinations. The method was extended to the study of the binding of small molecules to proteins by Cooper and Wood [60]. The use of soft gels limited the extension of this technique because of the low

flow-rates used and because of the gel shrinkage observed with large concentrations of protein solutions.

Now that rigid supports and HPLC techniques have been introduced, frontal analysis has gained in interest, as shown by several publications. The first HPLC application was given by Morris and Brown [61], who measured the binding of methyl orange to albumin using rigid beads and a medium range inlet pressure.

In the case of drug–protein binding studies, the applied eluent is a mixture of the interacting species. The chromatographic profile is in three parts (Fig. 4): a plateau (α) related to the unbound protein concentration; a region (β) due to the complex plus free species concentrations; and a region (γ) corresponding to the free ligand concentration. By carefully measuring the height of the last plateau one can determine the free ligand concentration and compute the ratio $\bar{r}$ (eqn. 1)

The advantage of the method is that it gives results at known and constant concentrations for all the species in equilibrium, the problems arising from protein self-association having been overcome.

We applied the method [62] to measure the warfarin–HSA binding using micro Bondagel® E 125 columns (Waters Assoc.) with high inlet pressures (100 bar). In this case, a drift in the last plateau signal was observed, which should correspond to the free ligand concentration. This effect results from a slow desorption process from the stationary phase that is detrimental for precise measurements. Moreover, repeated injections of large volumes of protein samples reduce the column lifetime. With a LiChrosorb diol (Merck) column better behaviour was obtained, since its adsorption properties towards proteins are low. The determination of diazepam–HSA binding [54] was possible with large concentrations of protein solutions (10 g l^{-1}). The results of these experiments reveal a decrease of the drug–protein affinity with increasing protein concentrations.

Recently we described a method that combines all the previous ones It was applied to the study of the binding bilirubin capacities towards plasma proteins

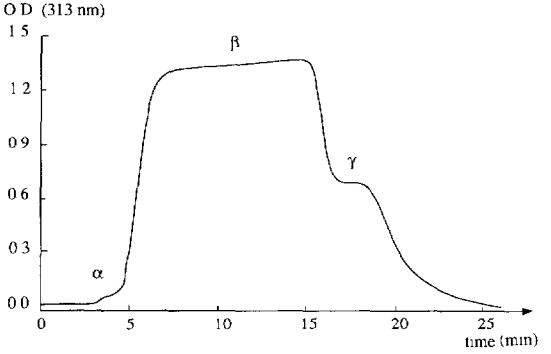

Fig 4 Elution profile in frontal analysis of a mixture of warfarin (10^{-4} *M*) and HSA (2 g l^{-1}) Eluent, warfarin (10^{-4}*M*) and HSA (2 g l^{-1}) in 0 067*M* phosphate buffer (pH 7 4). (Reproduced with permission from ref 38)

[63]. Because of the very low solubility of this ligand in water, both the protein and the ligand were dissolved in the eluent. When we injected a sample of a given protein and a known amount of bilirubin, we observed a first peak corresponding to the ligand–protein complex generally followed by a plateau due to the excess of ligand. An application of these experiments was the comparison of the bilirubin binding capacities of newborn with adult plasma proteins.

4.4. Vacancy peak method

In 1979, we introduced [64] a method based on an equilibrium saturation procedure using standard HPLC equipment. In a typical experiment, a size-exclusion colum is eluted with a solution containing a mixture of protein and drug in an aqueous buffer. After injection of a few microlitres of pure buffer, two negative peaks are detected at the column outlet (Fig. 5). The first peak corresponds to a vacancy in the ligand–protein complex, and the surface of the second one to the free drug concentration in the mixture. An appropriate calibration procedure (external or internal) gives again the ratio $\bar{r}$ and the binding equilibrium parameters. As the eluent contains the protein and the drug, the background absorbance is high and an internal calibration is recommended because of the non-linearity of the detector response. As in frontal analysis, the concentrations of free and bound species are kept constant during the whole chromatographic process, and this aspect is the main advantage of both methods. We applied the

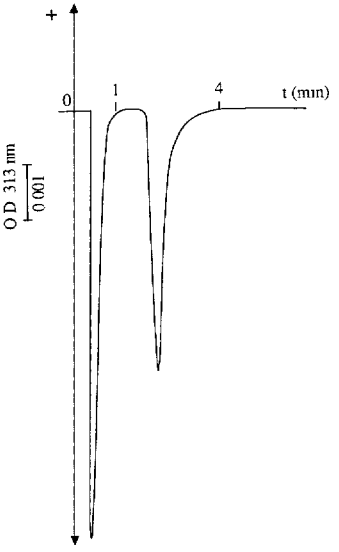

Fig 5. Vacancy peak chromatographic method Eluent, HSA (2 g l^{-1}) and diazepam (5 10^{-5} *M*) in 0 067 *M* phosphate buffer (pH 7 4). Injection volume, 50 μl of buffer, flow rate, 0 5 ml min^{-1}. (Reproduced with permission from ref. 54.)

equilibrium saturation method to study the influence of fatty acids on the stability of the warfarin–HSA complex [64].

The vacancy peak method is especially convenient for drugs that are weakly soluble in water. Since the presence of both the protein and the ligand in the eluent increases the ligand's solubility because of binding interactions, such measurements now become possible, whereas the Hummel and Dreyer method requires the complete dissolution of the drug in the buffer. Moreover, the equilibrium saturation or vacancy peak method is very useful to study competitive binding of several ligands to the same protein site, provided that the column can resolve the different components with an eluent containing the protein. Experiments based on the vacancy peak method revealed the role of sodium dodecylsulphate (SDS) on warfarin–HSA binding [65]. Because of its poor absorbance at the detection wavelength used, the SDS elution peak was detected indirectly by a signal resulting from the binding variations. This procedure gives information about the influence of SDS on the drug–protein interaction.

4.5. Retention analysis by zonal elution

This method has some common features with affinity chromatography, but does not require the protein to be immobilized on the chromatographic support: the mobile phase contains a given concentration of the protein in solution.

Using a LiChrosorb diol (Merck) column, the method was first applied to study the binding interactions of HSA to several drugs, such as warfarin, phenylbutazone, furosemide and *l*-tryptophan [66,67]. With these experimental conditions, HSA is practically excluded from the pores of the support. Owing to their small size the ligands to be studied penetrate the pores and appear with a retention larger than the void volume. The ligand retention may be explained by a partition or an adsorption mechanism due to the interaction of the drug for the stationary phase. This property is useful because it leads to better resolution between the drug and the protein peaks. With an eluent containing the protein, the drug retention is smaller than with the pure solvent. The affinity may easily be calculated from the retention difference. Nevertheless, some hypotheses have to be considered in theoretical treatments. The equilibrium isotherm characterizing the drug–protein interaction must be linear and therefore the ratio $\bar{r}$ must be high enough to be independent of the protein or drug concentrations. This may be checked experimentally by injecting drug samples of low enough concentration for constant retention times to be observed. Therefore, the method gives the global binding constant K' to the different classes of sites (eqn 2) Generally only the protein high-affinity sites are involved. This type of experiment permits the resolution of tryptophan enantiomers [67].

A similar method with reversed-phase chromatography extends the field of application. Marle *et al.* [68] have compared the results of binding measurements for the interaction of tryptophan or omeprazole with HSA using either LiChro-

sorb diol or reversed-phase RP8 from Merck, or phenyl Hypersil® from Shandon. The main difference between these last two supports and LiChrosorb diol is the irreversible adsorption of the protein on the reversed phases when an aqueous eluent is used. Owing to modification of the support surface by the adsorbed protein, a slight decrease of the drug retention volumes is observed An eluent containing the protein is used on this modified reversed-phase support and the retention volume of the injected drug is studied. From the variation of the drug retention with the protein concentration, the global binding equilibrium constant K' (eqn. 2) may be calculated. As outlined above, the ratio $\bar{r}$ (eqn. 1) must remain constant when increasing amounts of drug are injected into the column. In the case of non-ideal behaviour a dilution occurs during the elution transfer, which affects the ratio $\bar{r}$ and causes peak distortions with a concentration-dependent retention. Therefore, low amounts of drug have to be injected. With albumin in the mobile phase the same type of experiment has allowed the resolution of enantiomers and that of aromatic carboxylic acids [69] Their detection was improved with an indirect method based on the presence of a cationic chromophoric additive in the eluent. Similarly, tryptophan and omeprazole enantiomers have been resolved [68].

All the above methods used to determine drug–protein bindings do not require the use of any chemical immobilization of the species to be studied. The choice of the method depends mainly on the amount of material available. Because of the dissociation of the complex during the elution process, quantitation of drug–protein complexes by zonal chromatography is of limited application, and it is the other methods that are generally used. As they need eluents containing the drug and/or the protein, their use is likely to be limited by the amount of compounds spent in these experiments. Another drawback is related to the solubility of the ligand in the eluent. If the drugs are poorly soluble in water, it will be more convenient to choose methods in which the protein is dissolved in the eluent. However, chromatographic methods do not require the use of radiolabelled compounds as do dialysis techniques

5. AFFINITY CHROMATOGRAPHY WITH DRUG OR PROTEIN IMMOBILIZED

Affinity chromatography, one of the most powerful techniques for protein purification, is based on the molecular recognition properties between pairs of biological molecules. The technique relies on fundamental principles involving chemical equilibria between the species immobilized and those in solution. The method is specially suited for studying weak interactions with reversible equilibria, although for preparative applications, highly specific interaction are generally involved.

Reversible equilibria are generally found with drug–protein interactions [70], and affinity chromatography may then be used to measure the binding parameters. The method, now combined with HPLC [71], has gained in speed and

precision. To characterize quantitatively the interactions, two elution techniques are used [72], the frontal and the zonal elution modes, which differ in the volume of solute injected into the column.

In frontal analysis the adsorption equilibrium isotherm and thus the binding parameters of the interacting species may be directly obtained from a plot of the amount of solute adsorbed as a function of its concentration in the eluent Since the column reaches a quasi-equilibrium, frontal elution is a rigorous approach for measuring the interactions between free and immobilized species.

In linear zonal elution the input signal is a short pulse. When the amount of solute injected is low enough, the equilibrium isotherm may be considered as linear and the retention volume is proportional to the slope of the adsorption isotherm at the origin. The global binding constant K' (eqn. 2) may be determined from the value of the retention volume extrapolated to zero sample size, if the amount of active immobilized ligand is known. Pulse injection in linear affinity chromatography is the most popular method because of its simplicity and also because of the low amount of sample required. It has yielded valuable information, especially in competitive interaction and chiral recognition studies.

Zonal elution at finite concentrations may be used to determine the adsorption isotherm characterizing the interaction between species at equilibrium. The shape of the elution peak is studied as a function of the amount of solute injected (Fig. 6). The theoretical profiles are obtained by numerical simulations of the chromatographic process based on a given model of the equilibrium isotherm. Best fits of the theoretical model to the experiments yield the parameters characterizing the interaction of the solute with the immobilized species [73].

Affinity chromatography is used to study drug–protein interactions by immo-

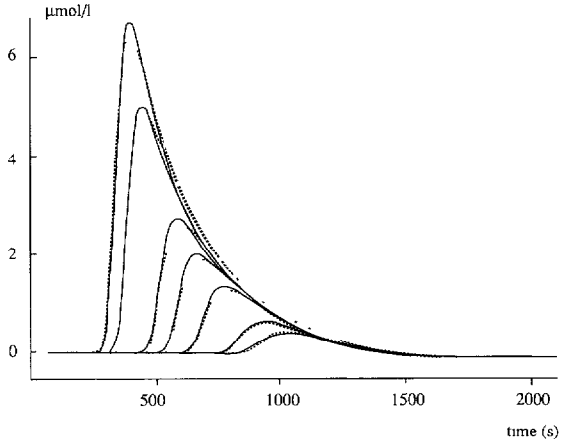

Fig 6 Affinity chromatographic zonal elution at finite concentrations on immobilized HSA Sample, 20 µl of phenylbutazone solution (18, 15, 9, 6, 4 and 2 10^{-5} *M*), eluent, 0 067 *M* (pH 7 4) phosphate buffer, flow-rate, 1 ml min^{-1}, temperature, 37°C Experimental ( ) and simulated (—) elution peak according to Scatchard model (Reproduced with permission from ref 73)

bilizing one of the interacting species on the chromatographic support. However, comparison with the constants measured in solution is questionable, since the chemical equilibria imply surface concentrations and involve interacting species that are different from those in solutions [72]. Measurements are generally carried out by immobilizing the protein on the chromatographic supports. The difficulty is then the sensitivity of the protein once it has been immobilized. Very few examples in the literature describe experiments with a drug immobilized on the chromatographic support. The point of attachment of the drug has then to be carefully chosen so that the drug can still bind with the protein.

5.1. Chromatography with immobilized drug

When a ligand is immobilized on the chromatographic support, a spacer arm must be interposed between the ligand and the matrix so that the properties of the immobilized ligand are as close as possible to those of the free one. The interaction between the protein and the immobilized ligand may differ considerably from their properties in solution, but free drug–protein associations may be studied from competitive experiments.

Veronese *et al.* [74] measures the interaction of psychoactive drugs with glutamate dehydrogenase (GDH) on a low-density perphenazine-Sepharose column. Competitive elutions of GDH were carried out with various buffers containing phenothiazines and butyrophenones The global dissociation constant characterizing the binding of psychoactive drugs in solution with GDH is obtained from the variation of the retention volume with the concentration of the drug in the eluent, if very small amounts of solute (GDH) are injected. The immobilized ligand and the drugs in solution compete for the same protein site.

Phenothiazine, an antipsychotic drug, and some analogues, were immobilized on Sepharose by Rochette-Egly *et al.* [75], in order to study the nature of the binding of these drugs with a calcium-binding protein, calmodulin. The binding of calmodulin to fluphenazine, perphenazine and 7-aminotriflupromazine involves specific electrostatic interactions and Ca^{2+}-dependent interactions. The method was applied for the purification of calmodulin from various tissues.

Strohsacker *et al.* [76] studied the interaction of cardiac myosin with sodium salicylate and showed that the protein interacts specifically with the immobilized drug. The ability of aspirin to bind specifically to cardiac myosin was used to purify the protein by HPLC on an affinity column derivatized with a solution of sodium salicylate.

5.2. Chromatography with immobilized protein

The binding of drug substances to proteins can be studied by affinity chromatography by immobilizing the protein on the chromatographic support. Lagercrantz *et al.* [77] were the first to demonstrate the potential of this method for

measuring drug–protein interactions. They outlined the various advantages of the method, which may be summarized as follows;

Easy determination and comparison of the binding properties of various drugs; possibility of evaluating the association constants of enantiomers if they can be resolved by the affinity column; convenient method for studying the competitive interactions with different ligands; reduced consumption of proteins in the case of zonal affinity studies, or with immobilized proteins if the same column can be used for a large number of experiments.

The interaction of some fatty acids, steroids and drug substances with bovine serum albumin (BSA) immobilized on Sepharose were studied at room temperature. The data were analysed according to a model assuming a protein with sites interacting with a ligand through several successive association constants. Frontal analysis was used to determine the association characterizing the interaction of salicylic acid with the immobilized BSA. The first or global association constant K' measured by frontal chromatography ($6.9 \cdot 10^4\ M^{-1}$) is in good agreement with that measured from zonal elution ($6.80 \cdot 10^4\ M^{-1}$). Frontal elution experiments were also analysed according to the Scatchard model, in which two different types of site are assumed. The number of sites of each type for the interaction of salicylic acid with BSA was determined as well as the corresponding association constants. To exploit zonal retention volume data, one has to evaluate the amount of immobilized protein. For this experiment, the amount of immobilized BSA was measured from the amount of digested albumin.

Several competitive experiments [77] for sites on immobilized BSA were also performed with salicylic acid and various labelled ligands. Some competition was found with clofibric acid, less with octanoate and no competition with oestradiol. Warfarin enantiomers are well resolved on this column and the resolution is increased when salicylic acid is added to the eluent. $R(+)$-Warfarin is more tightly bound to BSA than $S(-)$-warfarin.

From zonal experiments, Lagercrantz *et al.* [78] determined the first association constants of several labelled ligands (warfarin, tryptophan, salicylic acid) to serum albumins from various species. All proteins were able to resolve the enantiomers of DL-tryptophan. Warfarin was resolved into its enantiomers with all species except with baboon serum albumin. $S(-)$-Warfarin was more strongly bound than $R(+)$-warfarin to serum albumin of guinea-pig, human, rat and sheep, but the reverse order was found with chicken, cow, horse, pigeon and rabbit serum albumin. It is shown that chicken, guinea-pig and rabbit serum albumins bind salicylic acid very strongly.

The binding properties of HSA covalently immobilized on Sepharose were compared with those of the protein attached to blue Sepharose [79]. Alterations of the binding properties were found, since HSA stationary phase attached to Cibacron blue was no longer able to resolve enantiomers. The first association constants of various ligands (tryptophan, salicylic acid, warfarin, octanoate, decanoate and dodecanoate) were determined by zonal elution.

Using frontal elution. Nakano *et al.* [80] studied the interaction of salicylic acid with immobilized BSA. The binding properties of BSA covalently attached to an agarose gel matrix through a six-carbon-atom spacer were comparable with those of soluble albumin. The technique was further extended to study the simultaneous interaction of two drugs with immobilized HSA [81]. It was shown that sulphamethizole and salicylic acid compete for the same primary binding sites. Next, Nakano *et al.* [82] compared the binding properties of monomer and dimer HSA immobilized on Sepharose for salicylic acid, warfarin, phenylbutazone, mefenamic acid, sulphamethizole and sulphonylureas. They showed that the binding capacity of the dimer is *ca.* 10–30% less than that of the monomer. The binding of salicylic acid to HSA monomer immobilized on Agarose beads was studied at 4°C in Tris–HCl and phosphate buffers [83]. The large differences in the association constants were explained on the basis of competitive inhibition due to the binding of the chloride ions to the same active sites of HSA. Frontal affinity chromatography was also used to determine the drug–protein interactions by HPLC [84], on the basis of a Scatchard model. The association constants of warfarin, salicylic acid and diazepam with HSA immobilized to glyceryl porous glass beads were measured at 4°C.

Numerical simulations of the chromatographic process were applied by Vidal-Madjar *et al.* [73] to the determination of the equilibrium isotherm of drugs with HSA immobilized on diol-silica using zonal HPLC. It was shown that a three-parameter isotherm equation is adequate to characterize the binding of phenylbutazone to immobilized HSA and reveals two types of site on the protein with specific and non-specific interactions. The association constant of phenylbutazone with the high affinity sites of immobilized HSA at 37°C is about three times as large as the one characterizing the drug-free protein interaction. Zonal elution studies at finite concentrations offer a convenient way of determining the equilibrium isotherm and therefore the amount of active immobilised protein.

The usefulness of proteins for chiral recognition has been exploited by immobilizing them on HPLC supports, in order to resolve optical isomers. Recent reviews [85–87] have been published, and the results for drug chiral recognition on immobilized proteins will be briefly summarized here.

Several types of protein are used as chiral stationary phase on HPLC supports: BSA, α_1-acid glycoprotein (AGP), ovomucoid, α-chymotrypsin (ACHT) [87] and some of them are available commercially.

Allenmark *et al.* [87,88] have shown that BSA covalently bonded to silica HPLC supports is able to resolve many anionic enantiomers. This property was applied in the field of pharmacology to separate active racemic sulphoxides [89], β-adrenoreceptor blocking compounds [90] and racemic barbiturates [91]. Organic solvents added to buffer and their effect on drug retentions reveal the importance of hydrophobic interactions.

Hermansson [92] was the first to develop a chiral stationary phase with AGP bonded to silica. It has been shown that high separation factors can be obtained

for the enantiomers of basic drugs with different structures. Solvent and pH effects indicate that ionic as well as hydrophobic interactions take place with the immobilized protein.

Addition of charged or uncharged modifiers was used to regulate the retention and the stereoselectivity on the AGP columns [85]. Increasing concentrations of 2-propanol usually reduce the stereoselectivity, except with mepivacain and bupivacain. It is concluded that the enantiomers of these compounds are retained by a single type of chiral site. The retention and the selectivity of weak acidic drugs decrease with increasing concentrations of a cationic modifier, such as N,N-dimethyloctylamine. On the other hand, the retention of stronger acids is drastically increased [93].

The influence of mobile phase additives and pH on the retention and chiral resolution of various cationic and anionic drugs on an AGP HPLC column was studied by Schill *et al.* [94]. The retention of cationic compounds increases with pH, and the reverse is observed with anionic drugs. The influence of pH on stereoselectivity depends on the structure of the solute. The same authors also studied the elution properties of various ammonium drugs on AGP and the influence of various ionic and neutral modifiers added to the eluent [95]. The validity of a retention model based on an ion-pairing mechanism was demonstrated with cationic and anionic modifiers. These studies reveal that several different mechanisms seem to be involved in the interaction between AGP and drug molecules.

Aubel and Rogers [96] compared the chromatographic behaviour of various proteins immobilized on aminobutyl-derivatized silica, and the unique properties of BSA and AGP for chiral recognition were pointed out. The AGP column is able to separate optical isomers, but those that are resolved on the AGP column are not on the BSA column, and *vice versa*.

Miwa *et al.* [97] showed that ovomucoid, an egg protein, immobilized on silica is useful for the optical resolution of acids and amines by HPLC. The retention data demonstrate a strong hydrophobic interaction with basic solutes and a coulombic interaction with acidic compounds. The optical resolution of profen derivatives and other acidic drugs was achieved on an ovomucoid column [98].

Wainer *et al.* [99] recently introduced another type of biopolymer, ACHT, as a chiral stationary phase immobilized on an HPLC support. They studied the chiral recognition mechanism of a series of N- and O-derivatized amino acids on an ACHT column [100]. By blocking the active site of ACHT, it was demonstrated that other sites are responsible for hydrophobic, electrostatic and hydrogen-bonding interactions, and that chiral recognition of the solutes studied takes place at the hydrolytically active site of the enzyme.

These results show that zonal elution studies on immobilized protein offer a convenient way for rapidly characterizing drug–protein interactions by changing the nature of the solute and by exploring the influence of the eluent and that of various modifiers. The studies of the mechanisms for drug–protein chiral recog-

nition are of fundamental interest in molecular pharmacology, since optical activity may be directly related to the drug physiological effects.

6 CONCLUSION

In order to compare the different separation methods, the values of drug–protein binding constants are summarized in Tables 1 and 2, for two examples: the interaction of phenylbutazone and warfarin with serum albumin. The results are in most cases in good agreement and prove the validity of using chromatography as a tool for measuring the binding parameters of species interacting in solution.

The separation methods used to determine the drug–protein binding parameters must be selected according to the field of application.

For pharmacological studies such as clinical applications, the basic membrane procedures are generally used because they are especially convenient for concentrated protein solutions that contain a lot of different species. The drug quantitation is then very often based on radioactive labelling techniques.

Chromatographic methods applied to studies in solution are faster and gener-

TABLE 1

BINDING OF PHENYLBUTAZONE TO ALBUMIN

Comparison of the parameters determined from various separation methods

Method	n_1	k_1 (10^5 M^{-1})	n_2	k_2 (10^3 M^{-1})	$\Sigma n_i k_i$ (10^5 M^{-1})	Experimental conditions[a]	Ref
Equilibrium	1 5	2.3	3.7	5 6		2 g/l HSA, 37°C	101
dialysis	1 4	1.3	4.1	4		10 g/l HSA, 22°C	102
	5 9	1 3	3.3	5		1 g/l HSA, 22°C	102
	1	7 0	1	0 1		4 g/l HSA, 4°C	103
	1 3	5 1	8 1	5 6		2 g/l HSA	104
Dynamic	3 0	2 5	4 1	1 3		5 g/l HSA, 37°C	105
dialysis	3 2	2 8	4 2	2 3		5 g/l BSA, 37°C	105
Ultrafiltration	1 9	0 8			1.59	2 g/l HSA, 37°C	106
	1 2	19 2			22.0	HSA, 36°C	107
Hummel and Dreyer, HPLC	1.1	7 3	2 3	0.9		HSA, 37 °C	108
Retention analysis, zonal HPLC					8 5	0 01–1 g/l HSA, 37°C	66
Frontal affinity	1 6	2.1			3 29	Monomer ⎫ Immobilized	82
chromatography	1 5	1.7			2 65	Dimer ⎭ HSA, 4°C[b]	
Zonal affinity HPLC, peak-shape analysis	1	26 0			26 5	Immobilized HSA, 37°C	73

[a] pH 7 4, 0 067 *M* phosphate buffer, unless given.

[b] pH 7 87, Tris buffer and 0 1 *M* sodium chloride

TABLE 2

BINDING OF WARFARIN TO ALBUMIN

Comparison of the parameters determined from various separation methods

Method	n_1	k_1 ($10^5\ M^{-1}$)	n_2	k_2 ($10^3\ M^{-1}$)	$\Sigma\ n_i\ k_i$ ($10^5\ M^{-1}$)	Experimental conditions[a]	Ref
Equilibrium dialysis	1 0	2 3	3.7	0.6		2 g/l HSA, 37°C	101
	1	14	2	1 8		4 g/l HSA, 4°C	103
Dynamic dialysis	1	62 4	6	2.6		4 g/l BSA, 25°C[b]	109
	1 8	1.5	5.0	1 5		5 g/l HSA, 37°C	105
	2 1	1.1	5.0	1.6		5 g/l BSA, 37°C	105
Ultrafiltration	1 9	0.9			1 74	2 g/l HSA, 37°C	106
Hummel and Dreyer, HPLC	1 3	2 2	3 8	4.2		HSA, 37 °C	38
	1	2.5	4	3 9		HSA, 25°C	39
	1	3.6	4	8 3		BSA, 25°C	39
Vacancy peak, HPLC	1 3	2.2	3 8	4 2		0 01 g/l HSA, 37°C	64
Retention analysis, HPLC					3.25	0.01–1 g/l HSA, 37°C	66
Frontal elution: HPLC	1 2	2 1				2 g/l HSA, 37°C	38
Frontal elution: Gel permeation	1 4	2 0	3 7	2 2		3 g/l HSA, 37°C	110
Frontal affinity chromatography: Soft gels	2 1	0 87			1.81	Monomer HSA[c] 4°C	82
Frontal affinity chromatography: Soft gels	2 1	0 82			1 67	Dimer HSA[c] Immo-	82
Frontal affinity chromatography: HPLC	1 3	5 1	6 8	7 6		Monomer HSA bilized	83
Retention analysis, zonal affinity chromatography, soft gels					2 7	*S*-[^{14}C] Immobilized	77
					3 7	*R*-[^{14}C] BSA[d]	
					4.4	*S*-[^{14}C] Immobilized	78
					3 3	*R*-[^{14}C] HSA[d]	

[a] pH 7 4, 0 067 *M* phosphate buffer, unless given

[b] pH 7 3, 0 04 *M* phosphate buffer

[c] pH 7.87, Tris buffer and 0.1 *M* sodium chloride

[d] pH 7 4, 0 04 *M* phosphate buffer.

ally do not use the radiolabelling because of the improvements in detector technologies (UV, fluorescence). They are very useful to identify in a complex mixture the proteins that interact with a specific drug. The limitations of the technique arise either from sample dilution or from the eluent, since concentrated physiological solutions cannot be used in chromatography. Nevertheless, the high speed and accuracy of HPLC and the growing use of automatic equipment will lead to progress in the knowledge of the interactions between drugs and biopolymers

Affinity chromatography is of utmost importance to determine the thermodynamic and kinetic parameters. Moreover, the technique allows the isolation on a preparative scale of proteins with a specific activity towards drugs or metabolites, and this will certainly reveal essential information in immunopharmacology. Affinity chromatography has also led to considerable increase in the knowledge of the role of drug optical isomers in pharmacology.

7 SUMMARY

The review gives a critical evaluation of the different separation procedures used to study drug–protein interactions and describes their various fields of application.

For pharmacological studies, the most widely used methods are dialysis and ultrafiltration, because they allow measurements with solutions of high protein concentrations, such as those found in therapeutic conditions. Both techniques use membrane devices, which may induce additional binding effects. Another drawback of these techniques is the need for radiolabelled compounds.

Chromatographic methods, which now take advantage of the technology of high-performance liquid chromatography, are generally faster and do not use drug labelling because of the higher sensitivities of the detectors. Two different approaches are possible: either all the interacting species (protein and drug) are dissolved in the mobile phase, or one of them (protein or drug) is immobilized on the support.

Several chromatographic methods are available for studies in solution that differ according to the sample injection mode (frontal or zonal elution) and the nature of the mobile phase used. They include quantitation of the drug–protein complex by zonal elution, the Hummel and Dreyer method, frontal elution, the vacancy peak method, and retention analysis by zonal elution. Frontal elution is the most rigorous method since all the species at equilibrium are present in the mobile phase with known and constant concentrations. The most promising one is the Hummel and Dreyer method, because of the very small amount of protein injected in the mobile phase containing the drug.

Drug–protein interactions may be studied by affinity chromatography by immobilizing one of the interacting species on the support. Comparison of the constants obtained with methods when both the drug and the protein are in solution is questionable, since the immobilized species in affinity separations differ in their physical properties from those in solution. The main advantage with studies on immobilized proteins is the easy comparison of the binding properties of various drugs, especially when they are enantiomeric.

The results of the binding constants measured by different separation methods are given for the albumin–phenylbutazone and albumin–warfarin systems. Good agreement is generally obtained, which proves the validity of using chromatography as a tool to study drug–protein interactions.

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