J. Pharm. Pharmac., 1968, **20**, *Suppl.*, 150S–156S Received April 25, 1968

Protein-binding of small molecules: new gel filtration method

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The use of frontal analysis chromatography on Sephadex columns, previously described for protein-protein interactions (Nichol & Winzor, 1964) has been extended to the treatment of protein-small molecule interaction. Chromatograms obtained with bovine serum albumin and three different sulphonamides (sulphanilamide, sulphapyridine and sulphamethoxypyridazine) were found to conform to the theoretical patterns, and the values for the fraction of drug bound were in good agreement with those obtained by equilibrium dialysis.

THE binding of drugs to plasma and tissue proteins is an important factor affecting their distribution (Brodie, 1965) and rate of metabolism (Newbould & Kilpatrick, 1960; Anton & Boyle, 1964). As part of a program aimed at exploring any quantitative relation between protein-binding and the rate of drug metabolism, the use of gel filtration as a measure of protein-binding has been investigated. The widely used equilibrium dialysis method (Klotz, Walker & Pivan, 1946) requires prolonged equilibration times, with consequent risk of deterioration of protein in experiments at physiological temperature. Ultrafiltration (see, for example, Rehberg, 1943) is suspect because of possible changes in protein concentration during the experiment which would disturb binding equilibrium, although the use of small aliquots of filtrate overcomes this objection (Bennett & Kirby, 1965). Differential sedimentation in preparative or analytical ultracentrifuges (Büttner & Portwich, 1961; Cummings, Kuff & Sober, 1968; Steinberg & Schachman, 1966) although theoretically sound is not well adapted to making many routine measurements.

The use of gel filtration with Sephadex as partitioning medium has been explored by several workers. A frequently used procedure (Hardy & Mansford, 1962; Doe, Fernandez & Seal, 1962; Quincey & Gray, 1963) is to apply a small volume of protein ligand mixture to a column of Sephadex G-25 and to elute with buffer. The pore size of the stationary phase is such as to exclude proteins together with bound ligand, which therefore pass rapidly down the column, while admitting free ligand which slowly migrates as a separate zone. Since the two zones are completely separated this method is reliable only if the protein-ligand complex dissociates at a rate which is low compared with the rate of elution. Failure to appreciate this can lead to misleading results (De Moor, Heirwegh & others, 1962). This difficulty may be met (Hummel & Dryer, 1962) by applying protein-ligand mixture to a Sephadex column previously equilibrated with a solution containing the same ligand concentration as the mixture, elution being with the same solution. While results from this method have not been compared with those from equilibrium dialysis, it is theoretically sound, but extravagant of drug (Clausen, 1966). Several workers (Scholtan, 1964; Ashworth & Heard, 1966; Souleil & Nisonoff, 1968)

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have reported the use of Sephadex and similar gels in a batchwise manner, a known volume of solution containing known concentrations of ligand and protein being equilibrated with a known weight of dry Sephadex of suitable porosity. From the change of ligand concentration in the external (protein containing) phase the extent of protein-binding can be calculated provided the solvent uptake by the Sephadex is known. The method is equivalent to equilibrium dialysis but the composition of only one compartment, that containing protein, can be assayed with any precision. Together with the necessity to correct for adsorption of ligand onto Sephadex, this fact renders the method imprecise, particularly at low degrees of binding. We were therefore led to consider an alternative chromatographic method which appears to have none of the above disadvantages.

FRONTAL ANALYSIS OF PROTEIN LIGAND MIXTURES ON SEPHADEX COLUMNS

When a large volume of solution containing a reacting system, of the type $A + B \rightleftharpoons C$ is passed through a Sephadex column and then eluted

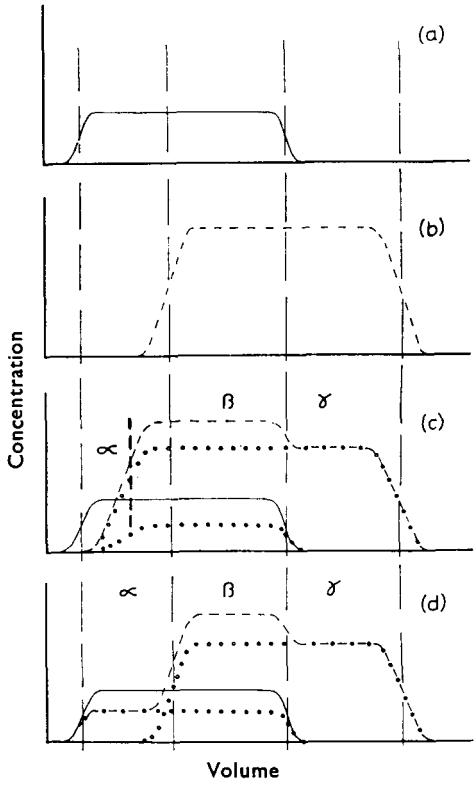

FIG. 1. Diagrammatic Sephadex chromatograms. (a) and (b) Pure reactants A and B respectively. (c) Equilibrium mixture of the rapidly reversible type. (d) Equilibrium mixture of the slowly reversible type. ——— total concentration of A; – – – total concentration of B; ○ ○ ○ ○ concentration of free B; ● ● ● ● concentration of C. Vertical lines indicate boundary positions.

with solvent the forms of the leading and trailing boundaries of the elution pattern depend on (*a*) the velocities, V_x, with which the various components move down the column, (*b*) the proportions of the three components and (*c*) the rates of the forward and reverse reactions.

When $V_A = V_C > V_B$ and the equilibrium is established rapidly compared with the rates of migration the form of the elution profiles is as shown diagramatically in Fig. 1c. Provided the velocities of the components are independent of the composition of the solution the positions of the various boundaries are related to those of pure reactants (Fig. 1a and b). It has been shown (Nichol & Winzor, 1964; Nichol, Ogston & Winzor, 1967) that under these conditions

$$C_B^\gamma = C_B^\beta,\ C_A^\alpha = \bar{C}_A^\beta \text{ and } C_C^\beta = \bar{C}_B^\beta - C_B^\gamma$$

where $\bar{C}_A^\beta = C_A^\beta + C_C^\beta$, $\bar{C}_B^\beta = C_B^\beta + C_C^\beta$

and C_A^α, C_A^β, C_B^β, C_B^γ, C_C^β are the molar concentrations of components A, B and C in the plateau regions α, β and γ of the chromatogram.*

If component A is a protein, component B a small molecular species and component C the protein-ligand complex, the fraction of ligand bound to protein in zone β (whose composition is identical to that of the starting solution) is

$$f = C_C^\beta/\bar{C}_B^\beta = (\bar{C}_B^\beta - C_B^\gamma)/\bar{C}_B^\beta \quad \ldots\ldots\ldots (1)$$

Many proteins form higher complexes with small molecules, of the type AB_2, AB_3 .... AB_n. Provided all these complexes migrate with the same velocity as C (independent of solution composition), and provided all the equilibria are rapidly attained, the above considerations are still valid. The number of moles of ligand bound per mole of protein in zone β is then

$$r = (\bar{C}_B^\beta - C_B^\gamma)/\bar{C}_A^\beta \quad \ldots\ldots\ldots\ldots\ldots (2)$$

The pore size of Sephadex G-25 is sufficient to exclude all proteins and drug-protein complexes (which would be expected to move with equal velocities) while admitting and consequently retarding small drug molecules. Some small organic molecules, particularly aromatic and basic substances, are reversibly adsorbed on Sephadex (Gelotte, 1960). Adsorption of proteins on the other hand appears to be negligible except at low salt concentration (Glazer & Wellner, 1962). Provided adsorption is reversible such factors will not affect concentrations in the plateau regions though they will affect elution volumes. Concentration dependence of adsorption coefficients will affect boundary shape. If the velocity of migration of one or more of the reacting components depended on the presence of the other, equations 1 and 2 would be invalid.

To test the validity of this approach to binding measurements the following experiments were made with serum albumin and several sulphonamides chosen to cover a wide range of extent of binding (Anton & Boyle, 1964).

*If the rates of the forward and reverse reactions are very slow compared with the rate of column flow the elution profile will approximate to that expected for three separate components (Fig. 1d) and $\bar{C}_B^\beta = C_B^\gamma + C_C^\alpha$.

Experimental

MATERIALS

Crystalline bovine serum albumin was obtained from Sigma, U.K. Ltd. Sulphanilamide (B.D.H. Ltd), sulphamethoxypyridazine (Lederkyn, Cyanamid of Great Britain, Ltd), and sulphapyridine (May and Baker Ltd), were used without further purification. Sephadex G-25 (fine grade) was obtained from Pharmacia Ltd. All other reagents were Analar or laboratory reagent grade.

SEPHADEX CHROMATOGRAPHY

Sephadex was allowed to swell in Sorensen's M/15 phosphate buffer, pH 7·0, at room temperature for 16 hr and packed into jacketed precision bore tubes (internal diameter 5 mm) to give columns 15 or 30 cm long. These were equilibrated for at least 4 hr with buffer at the required temperature (usually 37°) supplied at 12 ml/hr by a Buchler peristaltic pump. Sample solution (12 ml) was introduced through the pump and 5-drop fractions (approx. 0·3 ml) collected during sample introduction and subsequent elution with 12–15 ml buffer. Alternate fractions were assayed for protein and sulphonamide, correction being made for variation of drop size during the run.

EQUILIBRIUM DIALYSIS

Visking dialysis tubing (36/32 inch inflated diameter) was heated to 70° twice in distilled water and once in phosphate buffer and stored in buffer at 3° until required. Sample solution (20 ml) containing protein and sulphonamide was placed inside the dialysis tubing which, after closure, was immersed in buffer (80 ml) containing an amount of sulphonamide calculated to be approximately that expected in the external solution at equilibrium. The system was kept in a closed vessel at 3° for 48 hr. Equilibration was completed by agitating the vessel in a shaking thermostat bath at the required temperature (usually 37°) for 6 hr. Protein and sulphonamide were assayed in the internal and external solutions. To compare results from equilibrium dialysis and chromatography, samples of the equilibrated internal solution were passed through Sephadex columns.

Suitable control experiments showed that no protein passed through the Visking tubing during dialysis and that no material leached out of the tubing itself to interfere with protein or sulphonamide determinations.

Protein was estimated by the biuret method (Gornall, Bardawill & David, 1949) using bovine serum albumin as the standard: sulphonamides were assayed by a modification of the method of Bratton & Marshall (1939).

Results and discussion

Fig. 2 shows the results of a typical experiment in which the elution patterns of a number of mixtures of bovine serum albumin and sulphamethoxypyridazine are compared with the patterns obtained with the

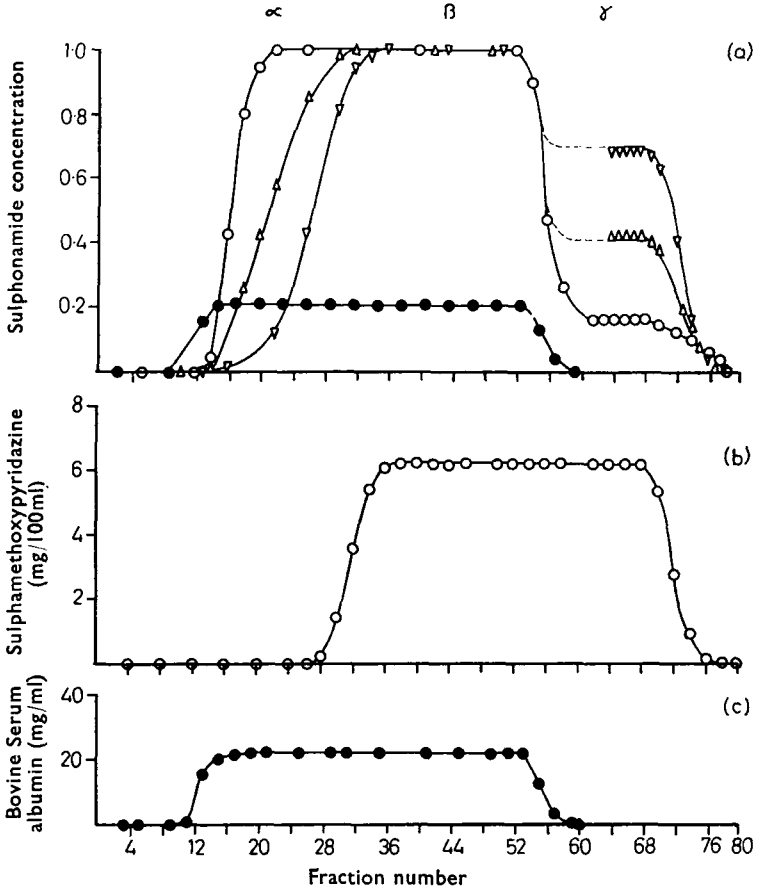

FIG. 2. Typical experimental Sephadex chromatograms. (a) Mixtures of sulphamethoxypyridazine and different concentrations of bovine serum albumin (○ 84%, △ 58% and ▽ 32% of drug bound), ● position of protein fronts in all three runs; in order to compare the results, the sulphonamide concentration for each run is expressed as a fraction of its value in the central plateau region β. (b) Pure sulphamethoxypyridazine. (c) Pure bovine serum albumin.

protein and sulphonamide separately. Particular features to note are:

(*a*) The positions of the leading and trailing boundaries of the protein zone are not affected by the sulphonamide. (In separate experiments they were also shown to be independent of protein concentration).

(*b*) The position of the slowest sulphonamide boundary is not affected by the presence of protein. (It was also shown to be independent of sulphonamide concentration).

(*c*) The sulphonamide boundary between zones β and γ coincides approximately with the trailing protein boundary but the leading sulphonamide boundary is in advance of the leading boundary for sulphonamide alone to an extent which depends on the composition of the mixture.

(*d*) The protein concentration is the same in zones α and β.

Similar results were obtained with sulphanilamide and sulphapyridine. Thus the form of the elution boundaries for these drug protein mixtures conforms to the simple theory for rapidly reversible equilibria and since in addition the composition of the solution in zone β was found to be identical with that of the original mixture (see also Table 1) the conditions necessary for the validity of equations 1 and 2 are satisfied.

Comparison of results obtained by equilibrium dialysis and chromatography (Table 1) shows that the concentration of sulphonamide in zone γ (C_B^{γ}) is the same as the free sulphonamide concentration estimated from dialysis and that values of r and f calculated from both sets of results are in good agreement. Frontal analysis thus provides a valid method for measurement of protein-binding, at least in cases such as the sulphonamides which, while showing some reversible adsorption on the Sephadex, have adsorption coefficients which are independent of concentration.

TABLE 1. COMPARISON OF BINDING RESULTS FROM DIALYSIS AND CHROMATOGRAPHY (for definition of symbols—see text)

Sulphonamide	Protein conc.* (mM)	Total sulphonamide conc. (mM)	Free sulphonamide conc. (mM) from dialysis	Chromatogram plateau conc. (mM) $\bar{C}_A^{\beta}$	$\bar{C}_B^{\beta}$	C_B^{γ}	r Dial.	r Chromat.	f Dial.	f Chromat.
Sulphamethoxypyridazine	6·6	23·5	16·0	6·8	23·6	16·1	1·13	1·11	0·321	0·319
	6·5	23·3	16·2	6·5	23·2	16·0	1·09	1·11	0·302	0·309
	14·0	19·8	8·1	14·2	21·1	8·7	0·84	0·87	0·590	0·584
	14·0	20·0	8·1	14·5	21·4	8·7	0·85	0·87	0·595	0·594
	29·7	28·7	7·5	30·0	28·3	7·2	0·71	0·71	0·739	0·748
	28·5	28·9	7·8	28·7	27·8	7·0	0·74	0·73	0·733	0·749
	29·7	27·9	7·4	28·7	28·5	7·2	0·68	0·74	0·735	0·750
Sulphapyridine	43·7	35·6	28·3	42·2	36·2	28·3	0·17	0·19	0·209	0·219
	44·1	35·2	28·1	41·8	35·2	28·1	0·16	0·17	0·207	0·209
Sulphanilamide	20·2	15·6	14·8	19·8	15·8	15·0	0·040	0·040	0·055	0·054
	19·9	16·2	15·5	19·8	16·3	15·7	0·035	0·030	0·048	0·041

* Molecular weight assumed—68,000.

In the present work the boundary and plateau regions of the chromatograms were evaluated in detail in order to validate the method but for routine purposes the procedure could be much simplified since the only information required is the concentration of the drug in the plateau regions. This fact would also make the method particularly suitable for continuous automatic monitoring of column eluate. It could also be scaled down considerably to reduce the minimum volume of sample required; this must always be sufficient, however, to give an overlap of protein and drug zones.

The method thus combines the advantages of speed, simplicity, economy of sample and reasonable precision. The possibility that analysis of the leading drug boundary can yield information about rates of formation and breakdown of drug-protein complexes (see footnote to p. 152 S), information which is not available from the other methods of measuring drug-protein equilibria, is being explored.

Acknowledgements. Gifts of sulphonamides from Cyanamid, Great Britain, Ltd. and May and Baker, Ltd., are gratefully acknowledged. This work was carried out during the tenure by one of us (P.F.C.) of a Medical Research Council Scholarship. We are also indebted to the Nuffield Foundation for financial support.

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