nevertheless still taken place. To confirm this, and because acetohexamide decomposes on melting, samples of the finely powdered crystals of polymorph B were scanned up to a temperature of 175°. On removal from the DSC pan, the powder was found by infrared examination to have undergone transition to form A.

Shenouda (1970) has noted that grinding can change a polymorphic form of sulphathiazole. With acetohexamide, however, the elimination of the transition peak by grinding may be due only to an effect on the thermal conductivity of the sample so that the transition takes place more gradually. It was not found possible to alter the infrared spectrum of polymorph B to that of A by grinding, and grinding in itself is therefore not sufficient to produce the transition.

The identification of polymorphic form in a commercially available sample of tablets containing 500 mg of acetohexamide per tablet was carried out by extracting a powdered tablet with 30 ml of water, to remove a yellow colouring material and any other water-soluble tablet excipient present, filtering and washing the residue well with water. The residue which was white in colour was dried in the oven at 105°. Its infrared spectrum was the same as that of polymorph A.

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REFERENCES

BALTAZAR, J. & FERREIRA BRAGA, M. M. (1966). *Revta port. Farm.*, **16**, 169–174.

BURGER, A. (1975a). *Sci. Pharm.*, **43**, 152–161.

BURGER, A. (1975b). *Ibid.*, **43**, 161–168.

BURGER, A. (1976a). *Pharmazeutische Industrie*, **38**, 306–312.

BURGER, A. (1976b). *Ibid.*, **38**, 639–643.

KUHNERT-BRANDSTÄTTER, M. & BACHLEITNER-HOFMANN, F. (1971). *Sci. Pharm.*, **39**, 15–28.

KUHNERT-BRANDSTÄTTER, M. & WUNSCH, S. (1969). *Mikrochim. Acta*, 1297–1307.

MEIER, G. N., KUHN, O. S., PIERART, F. O. & CORTES, S. J. S. (1971). *Revta R. Acad. Cienc. exact. fis. nat. Madr.*, **65**, 653–674.

SALIM, E. F. & HILTY, W. W. (1967). *J. pharm. Sci.*, **56**, 385–386.

SHAFER, C. E. (1972). *Analytical Profiles of Drug Substances*, **1**, pp. 1–14, Editor: Florey, K. London and New York: Academic Press.

SHENOUDA, L. S. (1970). *J. pharm. Sci.*, **59**, 785–787.

SIMMONS, D. L., RANZ, R. J., GYANCHANDANI, N. D. & PICOTTE, P. (1972). *Can. J. pharm. Sci.*, **7**, 121–123.

Estimation of the plasma protein binding of drugs by size exclusion chromatography at medium pressure (150 lb in^{-2}: 1·03 MPa)

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The most reliable method of estimating the degree of protein binding of a drug is to separate the unbound drug, before its assay, from the drug/protein mixture. However, the commonly used methods of separation suffer from the disadvantage that either the total drug concentration or the total protein concentration is altered during the separation process. For example, if a drug/protein mixture is dialysed, then the total drug concentration on the protein side of the membrane will decrease and a new equilibrium will be established. Similarly in ultrafiltration, the protein concentration will increase at the point of filtration, this being the point at which unbound drug is being separated from the mixture. Therefore a method is needed, in which it is certain that the concentrations of protein and total drug remain at their original concentrations, when the estimation of degree of binding is performed. A method which satisfies this criterion is frontal analysis using size exclusion chromatography (Nichol & Winzor, 1964; Cooper & Wood, 1968; Burke, 1969). The principle underlying this method is as follows (a complete treatment of analysis of results from frontal analysis is given by Nichol & Winzor, 1972). A column is packed with size exclusion material of pore size sufficiently small that only free drug can permeate into the pores while protein (and protein-bound drug) are excluded. A sample of the mixture to be analysed is applied continuously to the column. As the front moves down the column, unbound drug is retarded with respect to protein and protein-bound drug. To maintain an equilibrium, bound drug will then dissociate from the protein in the region not yet reached by unbound drug. At the void volume of the column (the only volume in the column available to protein), protein and remaining protein-bound drug will begin to elute. At a stage shortly after the elution volume of the unbound drug (the volume in the column available to unbound drug) has been reached the composition of the eluate will be identical to the composition of the sample, which is still being continuously applied. Under these steady state conditions the total concentration of drug (unbound + bound) can be determined by assay for drug in the eluate.

* Correspondence.

Then sample addition is terminated and the column eluted with buffer. Since the volume in the column available to unbound drug is greater than that available to protein, the protein will elute completely before the unbound drug. During this elution the equilibrium conditions will be maintained since protein will always be in contact with unbound drug at its concentration in the sample under analysis. After protein has been eluted, drug will continue to elute at the concentration of unbound drug in the sample, thereby allowing a direct assay of the unbound drug concentration. A major feature in favour of frontal analysis is that any non-specific binding sites for drug on the apparatus will be saturated with drug. The values obtained for the total and free drug concentration will thus be true values and will not be reduced due to adsorption of drug on the apparatus, as could occur for example in ultrafiltration.

The major drawback of this frontal-analysis size exclusion chromatography method is that with materials such as Sephadex only low flow rates can be achieved and so a single sample requires about 2 h chromatography time (Cooper & Wood, 1968). We have re-investigated this method using a non-compressible size exclusion medium at a pressure of 150 lb in^{-2} (1·03 MPa). As a model, the binding of methyl orange to bovine serum albumin in 0·07 M phosphate buffer (pH 7·4) was determined and the results compared with those obtained using equilibrium dialysis.

Size-exclusion chromatography. The apparatus consisted of a reciprocating pump with pulse damper (Jobling LD 711 eluant delivery unit), a Cheminert 6031 SVP rotary sample injection valve with a 10 ml sample loop of 1·6 mm i.d. PTFE tubing, a 20 cm × 4 mm i.d. glass column, a variable wavelength ultraviolet monitor (set at 280 nm) with a 10 μl flow cell and recorder. The connecting tubing was PTFE (0·58 mm i.d.) and the lengths between the sample valve and column inlet, and between the column outlet and flow cell were minimized to reduce mixing. The column was dry packed with Corning controlled-pore glass, (CPG 140) Glycophase-G size exclusion medium which has a particle size of 200–400 mesh (3·7–7·4 × 10^2 nm) and a nominal pore size of 4 nm. This material is coated with hydrophilic non-ionic carbohydrate to reduce non-specific adsorption and has a size exclusion range of 1000–8000 daltons, with reference to dextran in water. This means that methyl orange (mol wt 327 daltons) will permeate completely into the pores but bovine serum albumin (mol wt. 69 000 daltons) will be excluded. After packing, the column was eluted with buffer which had been filtered through a No. 4 sintered glass filter, and degassed.

During the size-exclusion chromatography, the pressure was maintained at 150 lb in^{-2} (1·03 MPa) producing a flow rate of 2·7 ml min^{-1}. All analyses were performed at 20°. The void volume, and the elution volume for methyl orange were found to be 1·5 ml and 5·0 ml respectively by chromatography of a mixture of 0·25% w/v blue dextran and 2 × 10^{-5} M methyl orange in filtered degassed buffer. Samples (21) containing 0·1% bovine serum albumin (Sigma, Fraction V) and concentrations of methyl orange ranging between 2 × 10^{-4} and 1 × 10^{-5} M were chromatographed as follows. The injection loop of the sample valve was filled with sample solution, using a syringe, with care taken to fully wash out the loop with sample. The valve was then rotated so the injection loop was connected between the pump and the column. After 6 ml of eluate had been collected the sample valve was rotated to disconnect the injection loop. This volume was chosen since it was 1 ml greater than the elution volume of methyl orange, so ensuring that steady-state conditions

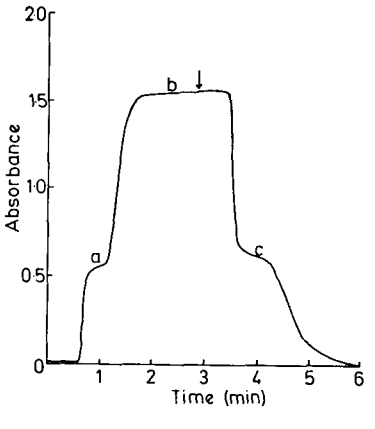

FIG. 1. Frontal analysis of 1·1 × 10^{-4} M methyl orange in 0·1% bovine serum albumin in 0·07 M phosphate buffer (pH 7·4)—The middle plateau (b) is the sum of the absorptions at 280 nm due to albumin and total methyl orange: the final plateau (c) is the absorption at 280 nm due to unbound methyl orange. Arrow indicates sample loop disconnected.

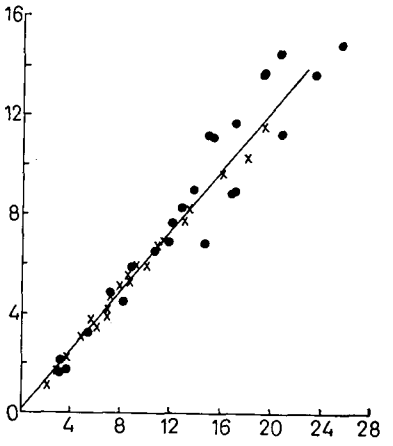

FIG. 2. Binding isotherm for the interaction of methyl orange with bovine serum albumin (pH 7·4). ●: results from equilibrium dialysis; ×: results from medium pressure frontal analysis size exclusion chromatography. The solid line represents the regression line for the combined data. Ordinate—[Unbound methyl orange] M × 10^5. Abscissa—[Total methyl orange] M × 10^5.

had been achieved. However, as the sample loop had not yet been emptied the desired sudden transition from sample to buffer took place without dilution of the trailing edge of the sample. Fig. 1 shows the result of a typical separation. The unbound methyl orange concentration was found from the absorption shown by the final plateau region (c, Fig. 1). The total methyl orange concentration was found by subtracting the absorption due to 0·1% albumin from the absorption shown by the middle plateau (b, Fig. 1), knowing that the extinction of methyl orange at 280 nm is not changed in the presence of 0·1% w/v bovine serum albumin. It was found that the total methyl orange concentration did not differ significantly from the concentration in the applied solution, confirming that steady-state conditions were achieved. The time required for each chromatographic separation was 10 min. A plot of unbound methyl orange concentration *vs* total drug concentration is given in Fig. 2.

Equilibrium dialysis. Equilibrium dialysis was performed at 20° using a Dianorm apparatus (MSE) with 1·2 ml capacity cells and Spectropor 2 membrane (this membrane has a cut off of 12 000–14 000 daltons). Solutions of methyl orange were prepared in buffer containing 0·1% w/v bovine serum albumin such that after dialysis the concentrations of free and bound methyl orange would be of the same order as for the size exclusion method. Preliminary studies showed that equilibrium was attained after 2 h dialysis and that there was no change in volume due to osmosis. Dialysis *vs* buffer was therefore performed for 2½ h. The solutions on either side of the membrane were then assayed for methyl orange at 470 nm by comparison with a calibration curve of absorption *vs* concentration of methyl orange in buffer or in 0·1% w/v bovine serum albumin as applicable. The results are shown in Fig. 2.

Analysis of data. There is a linear relation between the unbound methyl orange and total methyl orange concentrations over the concentration ranges chosen (Fig. 2) and so linear regression analyses were performed on the sets of data from each method and on the combined data. For the data from the size exclusion, the slope was found to be 0·583 ± 0·013 ($n = 21$, $r = 0.996$); for the equilibrium dialysis data, the slope was found to be 0·618 ± 0·040 ($n = 23$, $r = 0.958$) and for the combined data the slope was found to be 0·608 ± 0·022 ($n = 44$, $r = 0.973$). There is a better correlation and also a lower error with the medium pressure size exclusion method than with equilibrium dialysis. To compare the scatter of the data within the two sets with the scatter between the sets, the F ratio was calculated. The difference between the sum of squares of error for the combined data and for the data treated in two sets was found to be less than the sum of squares of error for the individual fits at the 95% confidence level, showing that the two samples belong to the same population.

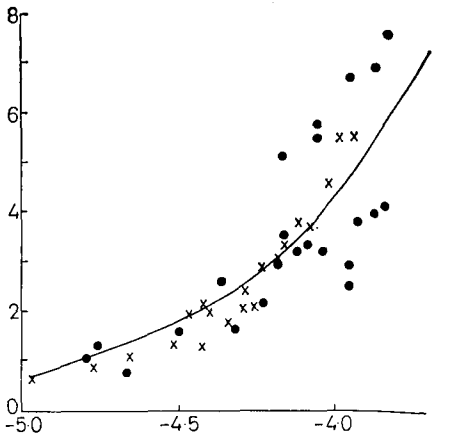

FIG. 3. Semi-log plot of the binding of methyl orange to bovine serum albumin; comparison of the data shown in Fig. 2 with literature data. ●: results from equilibrium dialysis (this work); ×: results from medium pressure frontal analysis size exclusion chromatography (this work). The solid line represents the binding curve quoted by Klotz, Walker & Pivan (1949). Ordinate—[Bound methyl orange]/[albumin]. Abscissa—Log [unbound methyl orange].

Conclusions. The size exclusion frontal analysis method yields results equivalent to those obtained by equilibrium dialysis and the results were found to be in good agreement with previously published data on the interaction of methyl orange with 0·1% bovine serum albumin (Fig. 3). This medium pressure size exclusion method is therefore an alternative to equilibrium dialysis for studying drug binding by physical separation of unbound drug from the drug/macromolecule mixture and it has two major advantages over equilibrium dialysis. Firstly, the degree of binding is measured directly for the solution under test, with no change in the equilibrium conditions. Secondly, a result can be obtained quickly without the problems of osmotic effects, denaturation, binding to apparatus and testing for attainment of equilibrium, which are inherent in the method of equilibrium dialysis.

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REFERENCES

BURKE, C. W. (1969). *Biochim. biophys. Acta*, **176**, 403–413.

COOPER, P. F. & WOOD, G. C. (1968). *J. Pharm. Pharmac.*, **20**, 1505–1565.

KLOTZ, I. M., WALKER, F. M. & PIVAN, R. B. (1949). *J. Am. chem. Soc.*, **68**, 1486–1490.

NICHOL, L. W. & WINZOR, D. J. (1964). *J. phys. Chem.*, **68**, 2455–2463.

NICHOL, L. W. & WINZOR, D. J. (1972). *Migration of Interacting Systems*, Oxford: Clarendon Press.