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The determination of the binding of salicylate to serum proteins

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The ratios of protein-bound to unbound salicylate in bovine, calf, horse and human sera were measured by equilibrium dialysis, ultrafiltration and frontal elution techniques. The equilibrium dialysis method gave the best measure of binding capacity, but several factors were found to affect the results. These factors include adsorption of the drug onto the dialysing membrane, the composition of the dialysing fluid, the necessity to allow sufficient time for equilibrium to be attained in the presence of serum, and the occurrence of dialysable substances in sera which may either compete with or displace salicylate from its binding sites.

A knowledge of the ratio of protein-bound to free, i.e. unbound, salicylate in the circulation is of obvious importance. The fraction of the drug in the unbound form is that available, at any one time interval, to enter the cells and initiate pharmacological and other actions. Some individuals show a reduced capacity to bind salicylate to their plasma proteins and may therefore be at higher risk after the ingestion of a toxic dose of the drug (Smith, 1968). Conventional laboratory methods (for example, Trinder, 1954) only measure the total salicylate concentration in the plasma and give no indication of the ratio between protein-bound and unbound drug. A convenient and reliable method for the separation and determination of protein-bound and unbound salicylate would therefore be of much practical value. It is relatively easy to assess such a method with respect to speed, simplicity and economy of sample. The evaluation of its precision and general accuracy is less straightforward. The usual practice is to compare it with either equilibrium dialysis or with ultrafiltration. The usefulness of the comparison depends on the reliability of these apparently established procedures as reference standards. They have been criticized (Cooper & Wood, 1968; Moran & Walker, 1968) because of possible changes in protein concentration due either to the prolonged manipulations or to absorption of the drug on to the dialysing membranes. We have therefore compared the results obtained from several sera and a purified bovine albumin fraction exposed to a range of salicylate concentrations, using ultrafiltration, equilibrium dialysis and frontal elution methods for the measurement of the ratio of protein-bound to unbound salicylate.

EXPERIMENTAL

Materials

Bovine albumin (fraction V) was obtained from the Sigma Chemical Co., St. Louis, and used as a 4% w/v solution. Horse serum, bovine serum and calf serum (natural clot, unheated) were obtained from the Wellcome Research Laboratories, Beckenham, and pooled human serum from the National Transfusion Service, Sutton. In some

experiments the sera were dialysed against the phosphate buffer before use; 50 ml samples of serum were dialysed against 1 litre of buffer, this being replaced three times over a 24 h period. Visking dialysis tubing (8/32 inch inflated diameter) was obtained from the Scientific Centre, London and G-25 Sephadex (medium grade) from Pharmacia Fine Chemicals, Uppsala. All chemicals were of analytical grade except for the sodium salicylate, which was of British Pharmacopoeial grade, and deionized water was used throughout. Unless otherwise stated, 0·1M phosphate buffer, pH 7·4, was used to prepare the solutions, to elute the gel columns and during the dialysis procedures.

Methods

Ultrafiltration was as described by Goldstein (1949) except that 1 ml of the reaction mixture was centrifuged at 3000 *g* and the first 50 μl of the ultrafiltrate used for analysis.

In the equilibrium dialysis experiments, the Visking tubing was soaked in two changes of water for 20 min before use. Sample solution (1 ml) inside the dialysis sac was dialysed against 10 ml of fluid in a vessel shaken 100 cycles per min on a Luckman rotary shaker for 20 h at room temperature (22°). Frontal elution was according to Cooper & Wood (1968); 7 ml of reaction mixture being applied to a 120 mm × 5 mm gel column and eluted with phosphate buffer. Salicylate was determined with an Aminco Bowman Spectrofluorometer, using an activating wavelength of 294 nm and a detecting wavelength of 413 nm.

RESULTS

Table 1 shows the results of experiments in which sufficient salicylate was added to various sera to give final concentrations of 50 μg/ml and the percentage of unbound salicylate measured by ultrafiltration, equilibrium dialysis and frontal elution. In some of the equilibrium dialysis experiments, the serum placed inside the sac was dialysed against a solution of salicylate in phosphate buffer sufficient to give a final concentration of approximately 50 μg/ml of salicylate inside the dialysis sac. The results with the sera showed wide variation. The results in Table 2 show that a prolonged soaking of the tubing (5 days) compared with a relatively short period (20 min), reduced the percentage of unbound salicylate measured by the equilibrium

Table 1. *Unbound salicylate in sera measured by different methods.* Each value represents the mean ± standard deviation of six determinations and is expressed as a % of the initial salicylate concentration (50 μg/ml) for the ultrafiltration and frontal elution methods. The results given for the equilibrium dialysis method represent the % of the final salicylate concentration (approximately 50 μg/ml), see text for details.

Serum	Equilibrium dialysis: protein-salicylate mixture inside sac	Ultrafiltration	Frontal elution
Bovine	4·3 ± 0·06	10·0 ± 0·16	10·8 ± 0·10
Calf	11·5 ± 1·60	41·7 ± 1·40	47·1 ± 0·30
Horse	7·3 ± 1·80	23·9 ± 0·30	22·0 ± 0·40
Human	1·0 ± 0·05	5·1 ± 0·10	5·0 ± 0·80

Table 2. *Adsorption of salicylate by dialysis tubing used in equilibrium dialysis method.* The Visking tubing was soaked in distilled water for either 20 min, with one change of water, or for five days, with 5 changes of the water. The lengths of open tubing were then exposed to 5 ml of either 10, 50 or 200 μg/ml salicylate solutions for 20 h and the salicylate concentration measured in appropriate solution at the end of this period. Each value represents the mean $\pm$ standard deviation of six determinations.

Length of tubing (cm)	Time in water	Salicylate concentration (μg/ml)		
5	20 min	10·1 $\pm$ 0·5	50·0 $\pm$ 1·3	202·9 $\pm$ 4·7
10	20 min	9·8 $\pm$ 0·4	50·7 $\pm$ 2·1	202·9 $\pm$ 6·7
20	20 min	9·7 $\pm$ 0·3	47·8 $\pm$ 3·7	197·0 $\pm$ 3·4
5	5 days	5·5 $\pm$ 0·2	47·0 $\pm$ 1·7	191·1 $\pm$ 4·9
10	5 days	1·4 $\pm$ 0·5	38·2 $\pm$ 3·0	182·2 $\pm$ 5·6
20	5 days	0·1 $\pm$ 0·1	18·9 $\pm$ 4·3	164·5 $\pm$ 5·6

Table 3. *Unbound salicylate in horse and human sera estimated by an equilibrium dialysis procedure with salicylate initially inside the dialysis sac, using different dialysing media.* Each value represents the mean $\pm$ standard deviation of six determinations and is expressed as a percentage of the final salicylate concentration (approximately 50 μg/ml).

Serum	Dialysing medium			
	0·1M Phosphate	Distilled water	0·9% NaCl	3·6% NaCl
Horse	12·9 $\pm$ 0·90	20·8 $\pm$ 0·70	32·0 $\pm$ 1·10	51·0 $\pm$ 7·10
Human	1·0 $\pm$ 0·05	0·6 $\pm$ 0·15	2·8 $\pm$ 0·14	5·9 $\pm$ 0·40

Table 4. *Unbound salicylate in sera measured by equilibrium dialysis method with salicylate initially either inside or outside the dialysis sac.* The results represent the percentages of the final salicylate concentration (approximately 50 μg/ml), see text for details. Each value is the mean $\pm$ standard deviation of six determinations.

Serum	Protein-salicylate mixture inside sac	Salicylate solution outside sac
Bovine	4·3 $\pm$ 0·06	4·3 $\pm$ 0·06
Calf	11·5 $\pm$ 1·60	22·7 $\pm$ 2·50
Horse	7·3 $\pm$ 1·80	10·4 $\pm$ 0·35
Man	1·0 $\pm$ 0·05	6·1 $\pm$ 0·40

dialysis method. The length of dialysis tubing subsequently used to make the dialysis sac also influences the results, but this effect only becomes apparent in the dialysis tubing which has been presoaked in water for the longer period. The results in Table 3 show the effect of varying the composition of the dialysing fluid in the equilibrium dialysis method. With the horse serum, the percentage of unbound salicylate increased when distilled water was substituted for the phosphate buffer and the values were even further enhanced when isotonic saline and hypertonic saline were used. The effects were similar, though less prominent, with the human serum,

except that virtually identical results were obtained with either distilled water or the phosphate buffer. Preliminary experiments showed that the dialysis in aqueous solution of salicylate in either direction in the equilibrium dialysis system was complete within 12 h. The results in Table 4 show that equilibrium occurred in the presence of bovine serum, but not in the presence of calf, horse or human sera, during a dialysis period of 20 h.

DISCUSSION

There are two aspects of the binding of drugs to circulating proteins. One is the capacity of a protein molecule to bind the drug; this is determined by the number of available sites. The second is the affinity of the drug for the binding site; this is determined by the dissociation of the drug-protein combination. There may be more than one type of binding site and the affinities for these may differ (see Davison & Smith, 1961). Both factors influence drug action. The binding capacity controls the pharmacological and other effects exerted at any one moment of time since these depend on the fraction of unbound drug available for entry into the cells. The affinity affects the rate of release of the bound drug from the circulating proteins and hence the duration of action. The separate determination of binding capacity and affinity for any drug-protein combination is desirable because these reflect different aspects of drug action. The methods used to investigate the binding of drugs to proteins are generally assumed to measure only the binding capacity but the results represent arbitrary combinations of binding capacity and affinity. The contribution of the affinity to the final result varies because the extent of dissociation of the drug-protein combination differs, depending on the experimental procedures employed. From this it can be inferred that the method which gives the lowest value for the amount of unbound drug has exerted the least effect on the dissociation of the drug-protein complex and gives the best measure of the binding capacity.

The results of the present work (Table 1) show that the equilibrium dialysis method meets this requirement for salicylate and the serum proteins of several species. However, using this method, several factors can influence the results. The first is adsorption of the drug to the dialysing membrane. Moran & Walker (1968) reported an appreciable adsorptive loss of salicylate from plasma samples into cellophane, and Goldstein (1949), working with methylene blue, commented that such loss may not only be large but variable from bag to bag. The present results (Table 2) also show that Visking tubing can adsorb salicylate. The extent of adsorption is principally affected by the nature of the pretreatment of the dialysing membrane. Thus, prolonged soaking (5 days) of the tubing in distilled water, compared with a short period (20 min) of soaking, caused a considerable increase in the adsorptive loss of the drug. Furthermore, with the tubing soaked for 5 days, the amount of salicylate lost from the drug solutions increased with the length of tubing eventually used to make the dialysis bag. The possibility of adsorptive loss on to dialysing membranes, and the influence of various forms of pretreatment of the membranes, must therefore be separately studied for each drug-protein or drug-serum combination.

A second factor is the composition of the dialysing fluid. The use of different media altered the values for unbound salicylate in horse and human sera and these alterations were not consistent (Table 3). Thus, substitution of distilled water for 0·1M phosphate buffer almost doubled the values with the horse serum but has the

reverse effect with the human serum. These results offer no guide to the choice of the most suitable dialysing fluid but only emphasize that the fluid used must be carefully described.

A third factor is whether sufficient time has been allowed, during an equilibrium dialysis method, to ensure that dialysis is complete and that equilibrium has been reached. A conventional method of testing this is to measure the time it takes for the drug, in aqueous solution, to reach equilibrium on both sides of the dialysing membrane. In the present work, preliminary experiments, in which the salicylate solutions were initially placed either inside or outside the dialysis sacs, showed that equilibrium was attained within 12 h. However, when experiments were repeated with various sera, the results (Table 4) showed that although equilibrium has been reached in the presence of the bovine serum, this has not occurred with the calf, horse and human sera, despite the dialyses being allowed to continue for 20 h. It must be concluded that data obtained from aqueous solutions of drugs cannot be used to predict the time necessary to reach equilibrium in experiments using different sera. Each combination of drug and serum must be separately investigated in order to establish this time when an equilibrium dialysis method is used to determine binding capacity.

A further factor may also be concerned because preliminary dialysis of the bovine serum before admixture with the salicylate reduced the values for unbound salicylate from 4·3 $\pm$ 0·06 (see Table 4) to 2·6 $\pm$ 0·02. When a purified bovine albumin solution was used instead of the bovine serum, the value was further reduced to 1·3 $\pm$ 0·06. One possible explanation is that other dialysable molecules, which could interfere with the binding of salicylate to the proteins, are present in varying amounts in the different sera. These molecules would be expected to be removed during dialysis of the sera or in the preparation of the purified bovine albumin fraction. It also follows that they may be removed to a variable extent during any method of equilibrium dialysis, the extent depending on the duration of dialysis and the ratio between the volumes of fluid inside and outside the dialysis sac. Thus, it is not possible because of these small molecules to obtain an absolute value for the binding capacity of serum proteins for a particular drug with any equilibrium dialysis technique. However, if the experimental conditions were such that the ratio of serum containing the drug to dialysate were high, then this would minimize the removal from the serum of any small molecules originally present which might interfere with the binding of the drug to the serum proteins. For example, if the ratio of 100 parts of serum plus salicylate to one part of dialysing solution were employed, then the value obtained for the binding capacity under these conditions would be similar to that existing in the circulation. This value, which for convenience could be termed the "actual binding capacity", may be of some clinical significance, since it is a good approximation of the *in vivo* binding capacity for salicylate at the time when the sample was collected. On the other hand, if the ratio of serum plus drug to dialysate was very low or if the serum sample was exhaustively dialysed before being analysed, then the small dialysable molecules would be largely removed and the value obtained would reflect the ability of the proteins to bind the drug only. Thus if, in the equilibrium dialysis technique, the ratio of serum to dialysing fluid was 1 to 100, then a measure of what may be termed the "potential binding capacity" of the serum sample for salicylate would be obtained. Many of the published techniques for estimating binding capacity by equilibrium dialysis methods measure neither actual nor potential

binding capacity but an intermediate value between the two. It is possible to estimate them separately as described above and this may be of some relevance in serum samples from patients receiving multiple drug therapy.

Although the results of the present work suggest that equilibrium dialysis is the method of choice for determining binding capacity, it must be stressed that there are several factors which influence the results obtained by this method and they must be separately investigated for each drug-protein or drug-serum combination being studied.

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