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A rapid method for the determination of salicylate binding by the use of ultrafilters

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An ultrafiltration method that combines speed, simplicity, accuracy and precision was employed to measure plasma binding of salicylate. Human plasma, containing 1·0 and 0·1mM sodium salicylate, was ultrafiltered through membranes using 45 p.s.i. and slow rotational stirring. Salicylate was bound at these concentrations 13·1 and 4·4% respectively. This method, using membranes that quantitatively separate small molecules from plasma proteins, yields results comparable to those obtained by equilibrium dialysis.

In an analysis of the methodology used to measure the binding of salicylate to serum proteins, McArthur & Smith (1969) state "A convenient and reliable method for the separation and determination of protein bound and unbound salicylate would be of much practical value." They suggest this method must have "speed, simplicity and economy of sample." It also must reflect the situation *in vivo;* i.e., it must be *precise* as well as accurate. Moreover, McArthur & Smith (1969) state, "From this it can be inferred that the method which yields 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." Using these criteria, they conclude that equilibrium dialysis is the method of choice, although at least 20 h are necessary for equilibration.

The recent manufacture and commercial availability of ultrafilters that allow quantitative separation of proteins from smaller molecules, and an apparatus that with pressure and slow rotational stirring allows a satisfactory rate of filtration, suggested to us the use of these ultrafilters in drug-binding studies. The following report is an analysis of this potential method to evaluate the binding of salicylate to fresh human plasma.

MATERIALS AND METHODS

[^{3}H]Salicylic acid (specific activity: 100 mCi/mmol) and [^{14}C] 3-*O*-methyl-D-glucose (specific activity: 10 mCi/mmol) > 98% pure by chromatographic analyses were obtained from New England Nuclear Corporation. ^{125}I labelled human serum albumin (specific activity of 9·6 μCi/mg) was obtained from Mallinkrodt Chemical Works. Sodium salicylate was of pharmaceutical grade. All filtrations were run on Millipore 25 000 Pellicon filters (Cat. no. PSED02510).

The filtration apparatus, a prototype from the Millipore Corporation, consisted of a 10 ml plastic cylindrical chamber into which the filter was placed, with the Pellicon membrane surface up, on a stainless steel mesh. A Teflon-coated magnetic stirrer was placed inside and a 10 ml sample was added to the cleaned, dry chamber which was then capped and a pressure of 40 to 60 p.s.i. of 100% nitrogen was applied via the cap

to the chamber. The magnetic stirrer was set to approximately 100 rev/min. The filtrate ran through the filter into a continuous groove in the base of the apparatus to the outside. The volume of fluid below the membrane was approximately 0·3 ml. The filtration of 2 ml of plasma filtrate or whole blood filtrate required approximately 25 min. After each filtration the filter was discarded and the apparatus was washed in distilled water and dried thoroughly.

To confirm the exclusion of protein in the filtrate, ^{125}I labelled albumin was added to 10 ml of a balanced salt solution (Merlis, 1940) or to 10 ml of plasma and filtered. The filtrate and the parent solution of each were counted in a standard, well-type sodium iodide gamma scintillation counter at 35% efficiency.

To demonstrate the quantitative recovery of salicylic acid and 3-*O*-methyl-D-glucose in the absence of protein, approximately 0·01 μmol of tritium labelled salicylic acid and 1·0 μmol of [^{14}C] 3-*O*-methyl-D-glucose were added to 8·5 ml of saline and 1·5 ml of Merlis solution and shaken in a Vortex mixer. This solution was filtered and the first ml discarded because of slight binding, particularly of ionic compounds, by the cellulose base of the membrane. Approximately 0·5 ml of filtrate of an 0·004 mM sodium salicylate solution saturates these sites (Fig. 1). Fifty μl aliquots of the parent solution and filtrate were counted in 10 ml of a Triton-X-100 based scintillation

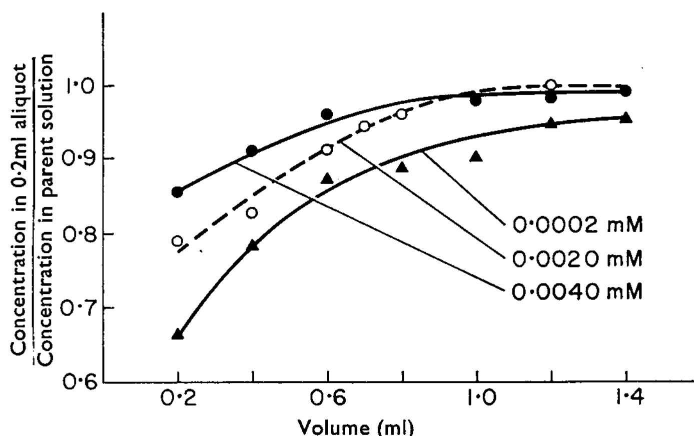

FIG. 1. Saturation of binding sites below filtration membrane as a function of volume of filtrate and salicylate concentration.

fluor in a Packard Tri-Carb liquid scintillation counter using standard methods (Branscombe, 1970) for double isotope counting, quench correction and isotope ^{14}C spill correction.

To determine plasma binding, fresh heparinized plasma was obtained from a single male donor. Immediately after centrifugation, trace quantities of [^{3}H]salicylic acid were added to 8·5 ml plasma and 1·5 ml Merlis solution with sufficient carrier sodium salicylate to make the solution 0·1 mM sodium salicylate. The mixture (the parent solution) was shaken in a Vortex mixer and allowed to stand at 23° for 1 h and then filtered. The first 1 ml of filtrate was discarded, the apparatus opened and 50 μl of parent solution withdrawn and counted. The apparatus was closed, and 2 $\times$ 0·5 ml aliquots of the filtrate were then collected and 50 μl from each removed and counted. Then the apparatus was reopened and 50 μl of the parent solution were withdrawn

and counted. Free salicylate was calculated after correction for quench and ^{14}C spill by dividing the average counts from the $2 \times 0{\cdot}5$ ml filtrate samples by the average counts in the parent solution before and after the filtration of the $2 \times 0{\cdot}5$ ml aliquots.

On three occasions, whole heparinized blood was filtered in the same way as the plasma except that 9 ml of blood was taken; 0·5 ml of blood was removed at the initial supernatant withdrawal, and 0·5 ml removed at the end. The haematocrit was taken on both samples. Also, 50 μl of whole blood were counted as well as 50 μl of plasma from the blood after centrifugation of these two samples.

On two occasions, the salicylate in the parent solution and filtrate was determined chemically (Routh, Paul & Dryer, 1956).

The binding of salicylate to plasma was determined by the gel filtration method of Smith & Sturman (1967) using the above radiochemical method instead of fluorometric analysis to determine salicylate concentrations.

RESULTS

Plasma protein was quantitatively excluded by the Pellicon membrane by the criteria of: (1) inspection of the filtrate which was crystal clear, (2) exclusion of [^{125}I] labelled human serum albumin and (3) ultraviolet absorption as carried out by the manufacturers of the apparatus.

Recovery of salicylate and 3-*O*-methyl-D-glucose was quantitative when the first ml of filtrate was discarded when no plasma was present. Recovery of 3-*O*-methyl-D-glucose was quantitative in the presence of plasma when correction was made for the plasma protein concentration. At 1 mM sodium salicylate, $13{\cdot}1 \pm 0{\cdot}8$ (s.e.)% ($n = 6$) was unbound in the plasma. At 0·1 mM sodium salicylate, $4{\cdot}4 \pm 0{\cdot}4$ (s.e.)% ($n = 4$) was not bound.

At 1 mM sodium salicylate, whole blood yielded 26% unbound salicylate; but when this was corrected for red cell volume, the yield was 15% unbound salicylate with respect to plasma. These results indicate the red cells contain approximately the free concentration in the plasma.

The chemical method (Routh & others, 1956) results showed 16% unbound on two samples compared to a radiochemical result of 14% unbound on the same samples at 1 mM plasma concentration of carrier sodium salicylate. The gel filtration method of Smith & Sturman (1967) yielded extrapolated values of 15% unbound at 0·1 mM salicylate and 35% unbound at 1·0 mM salicylate. These values correspond closely to the values for the binding of salicylate by human plasma as determined by gel filtration (Smith & Sturman, 1967).

DISCUSSION

McArthur & Smith (1969) compared the binding of salicylate in bovine, calf, horse and human serums using the methods of equilibrium dialysis, ultrafiltration and frontal elution (a gel filtration method). Using the criterion that "the method which gives the lowest value for the amount of unbound drug . . . gives the best measure of the binding capacity," they concluded that, of the three methods, "equilibrium dialysis is the method of choice for determining binding capacity." Cooper & Wood (1968) in their evaluation of frontal elution state that gel filtration (e.g., as performed by Smith & Sturman (1967) "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

could lead to misleading results." This phenomenon, essentially a dilution, may explain the low values of salicylate binding obtained by gel filtration studies.

Moran & Walker (1968), using radiochemical methods and ultra-filtration through Cellophane, found a "definite adsorptive loss" on the Cellophane. "Only after filtration of one ml" or 20% of their sample did the ultrafiltrate reach the starting value of aqueous standards. Thus, the removal of initial aliquots of ultrafiltrate through Cellophane would yield low values for salicylate binding.

Our method, using ultrafilters, combines the advantages of speed, simplicity, accuracy and precision. At present, 8·5 ml of plasma is required, which does not yield economy of sample. The difficulty could possibly be circumvented by saturating the binding sites in the cellulose below the filter so that the initial aliquot from the apparatus could be compared directly with the parent solution.

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