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The binding of indomethacin, salicylate and phenobarbitone to human whole blood *in vitro*

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The binding of indomethacin, salicylate and phenobarbitone to human whole blood, plasma and red cells has been determined by equilibrium dialysis. The red cells bound appreciable proportions of salicylate and phenobarbitone but not indomethacin. It is concluded that prediction of the extent of the drug binding to the circulating blood should be made from the results obtained with whole blood, red cells and plasma.

After entering the blood a drug may circulate in a bound form. The bound drug can be regarded as a storage depot since only the unbound fraction is able to penetrate the cells and initiate pharmacological actions. In addition, when a drug binds to the circulating proteins it may displace other small molecules, such as amino-acids and fatty acids (McArthur & Dawkins, 1969; Dawkins, McArthur & Smith, 1970), from their binding sites on the proteins. A common practice is to determine the binding of drugs to either separated plasma or to purified albumin fractions and to use the results to predict the circulating levels of bound and unbound drug. Albumin has been shown to be responsible for most plasma protein-drug interactions although some of the other proteins may also be concerned. The possible influence of the erythrocytes and other cellular components on drug binding in blood has been largely neglected. The present investigation is concerned with comparing the binding characteristics of indomethacin, phenobarbitone and salicylate in human whole blood and plasma *in vitro*.

MATERIALS AND METHODS

Materials

Samples of blood (200 ml) were obtained by venepuncture from healthy normal males and collected into plastic bags, 1 ml of heparin containing 1 mg sodium heparin (100 IU) being used as an anticoagulant. Approximately 150 ml of each sample of whole blood was centrifuged at 3000 *g* for 15 min and the separated plasma divided into two equal portions. One of these (undiluted plasma) was used as such and to the other (diluted plasma) was added sufficient 0·9% (w/v) NaCl solution to adjust its volume to that of the corresponding volume of whole blood from which it had been prepared. A similar procedure was used with the red cells separated by the centrifugation except that these were suspended and centrifuged in six quantities, each of 100 ml of 0·9% (w/v) NaCl solution, before the final volume was adjusted to that of the initial sample of whole blood (diluted red cells). No attempt was made to separate either the leucocytes or blood platelets and the red cell fraction includes both these components. Visking dialysis tubing (8/32 inch inflated diameter)

was obtained from the Scientific Centre, London, and powdered indomethacin was a gift from Merck, Sharp & Dohme Ltd., Hoddesdon, Herts. All chemicals were of analytical grade except for sodium phenobarbitone and sodium salicylate, which were of British Pharmacopoeial grade, and distilled water was used throughout.

Measurement of drug binding

Aliquots (2 ml) of either whole blood, plasma, diluted plasma or diluted red cells were placed inside sacs of Visking tubing which had been soaked in two changes of water for 20 min before use. They were dialysed against 3 ml of 0·9% (w/v) NaCl solution containing sufficient of the drugs to give initial concentrations as follows: phenobarbitone, 0–3 mM, indomethacin, 0–1 mM and salicylate 0–10 mM, in vessels shaken at 100 rev/min for 28 h in a water bath at 8°. The indomethacin was suspended in distilled water and sufficient N NaOH added to yield a 5 mM solution of the drug. Sufficient 0·9% (w/v) saline was added to give the range of indomethacin concentrations required and in each case the final solution was adjusted to pH 7·2 by the addition of 0·1N HCl. Preliminary experiments were made to ensure that no loss of drug occurred during dialysis due to adsorption to the tubing and each combination of drug and blood fraction was separately investigated to establish that dialysis was complete and equilibration attained within 28 h. Indomethacin and salicylate were estimated in samples taken from outside the sacs with an Aminco Bowman Spectrophotofluorimeter using activating wavelengths of 290 and 294 nm and detecting wavelengths of 385 and 413 nm respectively. The phenobarbitone concentration was determined by adjusting to pH 10·0 with 0·1M borate buffer and measuring the absorption at 240 nm using a Unicam SP800 spectrophotometer. The concentrations of the drugs outside the sacs at the end of dialysis are the unbound concentrations. At the end of the dialysis the concentrations of the drugs inside the sacs containing the blood preparations were calculated by subtracting the amounts of drug outside the sac when dialysis was complete from the total amounts of drug, i.e. inside and outside the sacs, found in the corresponding experiments when only saline was inside the sac. The concentrations of bound drugs inside the sacs at the end of dialysis were obtained by difference.

RESULTS

The binding curves of indomethacin to whole blood, plasma, diluted plasma and diluted red cells are shown in Fig. 1. In this and the subsequent figures the concentrations of unbound drug have been plotted against the concentrations of bound drug. The results with indomethacin show that the drug is not bound by the red cells but that at a total concentration of 0·2 mM approximately 75% is bound by the plasma. The binding curve for whole blood is almost identical to that of the plasma to which has been added a volume of saline equivalent to that of the erythrocytes (diluted plasma). Thus the contribution of the red cells to the binding of indomethacin is negligible.

The binding curves for salicylate (Fig. 2) and phenobarbitone (Fig. 3) differ from those of indomethacin in that appreciable proportions of both drugs are bound by the red cells and the plasma. Measurements of the binding to separated plasma *in vitro* would suggest that the free, i.e. unbound concentrations of the drugs are smaller fractions of the total concentrations that would be present *in vivo*.

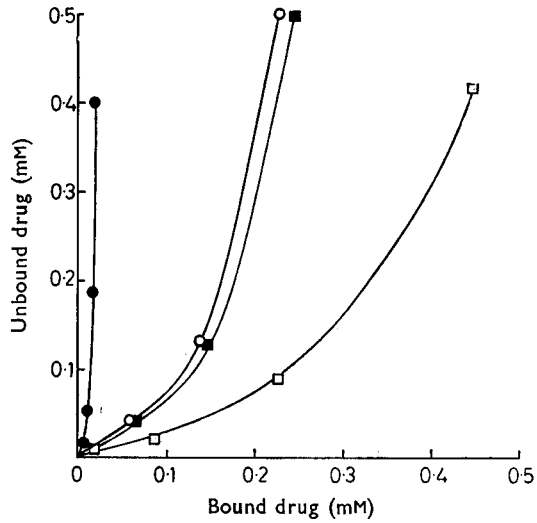

FIG. 1. Binding curve of indomethacin to blood fractions *in vitro*. ● Diluted red cells. ○ Whole blood. ■ Diluted plasma. □ Plasma.

DISCUSSION

The study of the binding of drugs in the circulation has conventionally been restricted to their interactions with plasma proteins. One reason is the obvious convenience of experimental techniques using either separated plasma or purified protein fractions. The availability of highly purified albumin preparations has provided an impetus to the detailed investigation of the binding sites and nature of the forces which cause drugs to interact with plasma albumin. Other plasma proteins can and do participate in drug binding but the possible influence of the cellular constituents in the blood have received scant attention (Goldstein, 1949; Meyer & Guttman, 1968).

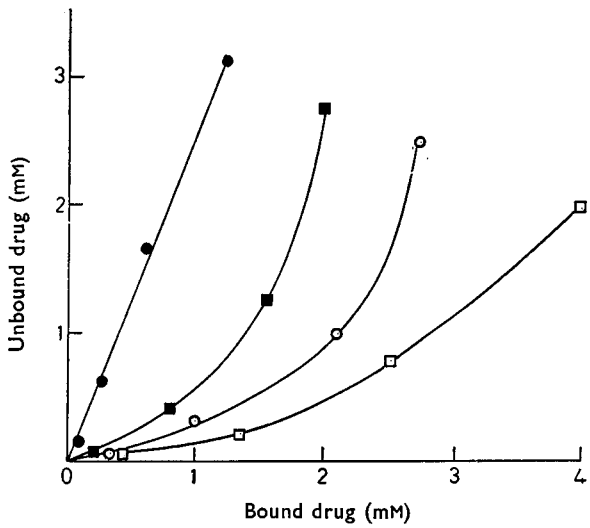

FIG. 2. Binding curve of salicylate to blood fractions *in vitro*. ● Diluted red cells. ○ Whole blood. ■ Diluted plasma. □ Plasma.

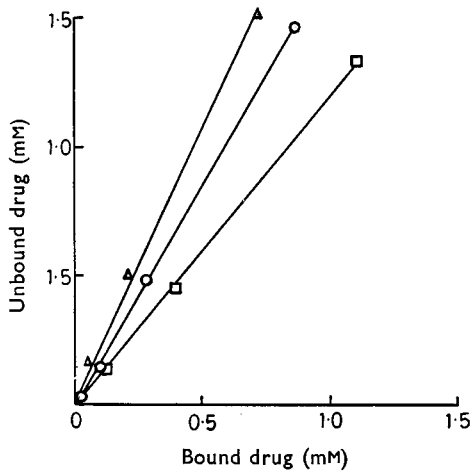

FIG. 3. Binding curve of phenobarbitone to blood fractions *in vitro*. △ Diluted red cells and diluted plasma. ○ Whole blood. □ Plasma.

The present results show that for three widely used drugs, indomethacin, salicylate and phenobarbitone, the determination of their binding to separated human plasma would yield misleading information about the ratio of bound to unbound drug in the circulation. It is the fraction of drug in the unbound form which is available, at any one time interval, to enter the cells and initiate pharmacological and toxic actions. The determination of this fraction is of obvious importance in relating *in vitro* effects to *in vivo* actions, in the assessment of the efficiency of a drug in different formulations and after various rates of administration, and in the investigation of untoward effects. Thus an individual may be at a higher risk after the ingestion of a particular dose of a drug because of a reduced capacity to bind the drug in the circulation.

The present results show that human red cells do not bind indomethacin. It would be possible to predict values for unbound indomethacin in the circulation from a plasma binding curve for the drug prepared *in vitro* if appropriate corrections were made for the volume occupied by the red cells, i.e. diluted plasma binding curve. This situation does not apply to salicylate and phenobarbitone since both these drugs bind to the red cells. It is necessary to measure their binding curves to whole blood rather than to either separated plasma or purified albumin fractions. The present results with salicylate do not agree with either those of Coburn (1943) who stated that the drug was excluded from human red cells or of Lester, Lolli & Greenberg (1946) and Smith, Gleason & others (1946) who concluded that the red cell was freely permeable to salicylate but did not bind the drug.

The concentrations of indomethacin and salicylate used in the *in vitro* experiments were equivalent to those observed in man either during therapy or in acute intoxication (Hucker, Zacchei & others, 1966; Smith, 1966). The plasma concentrations of phenobarbitone after medicinal doses is about 0·2 mM and concentrations up to 0·5 mM have been reported in acute poisoning (Broughton, Higgins & O'Brien, 1956). The range of concentrations of the drug used in the present work was higher because of the relative insensitivity of the analytical method at very low concentrations.

It is concluded that the determination of the extent of binding of a drug to either separated plasma or to purified protein fractions provides inadequate data about the binding of drugs in the circulation. The interaction of the drug with red cells should also be measured. If a drug binds to red cells, then the diluted plasma binding curves yield lower figures for bound drug than would be obtained with whole blood *in vitro* and expected to occur in the circulating blood. It is possible that a drug may not bind to plasma proteins but bind to the red cells. The determination of its binding to plasma or to albumin preparations would yield results suggesting that it would leave the circulation at a rapid rate whereas its complex with the red cells might enable it to persist for a considerable period. Studies of drug binding *in vitro* should therefore be made with whole blood in addition to any measurements performed on plasma or separated plasma protein fractions.

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