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The extraction of azovan blue from precipitated plasma proteins

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The paper describes a method for the estimation of azovan (Evans) blue in plasma proteins precipitated by trichloroacetic acid. The precipitate is dissolved in a strong solution of urea and an anionic detergent; the dye is absorbed on a cellulose column, from which it can be eluted by alkaline acetone-water. The method allows information to be obtained about drug uptake and plasma volume without the need for additional blood samples. It has been used with cat, rabbit, rat and guinea-pig plasma.

In the course of studies on the uptake of choline from the circulation of cats and rabbits, azovan (Evans) blue (T-1824) was added to the solutions of choline administered to act as unabsorbable tracer. Free choline in the plasma was estimated essentially by the method of Bligh (1952) and it was noticed during the precipitation of the proteins by trichloroacetic acid that the dye was retained by the precipitate. Since the problem of blood loss is always troublesome with small animals when repeated blood samples are taken, the possibility of extracting the dye from the precipitate was investigated. It was found that the proteins were soluble in strong urea solution and that the dye could be removed from the resulting solution by absorption onto cellulose. The method described below permits azovan blue and free choline to be estimated on the same sample of the plasma and thus halves the volume of blood required.

EXPERIMENTAL

Materials

Urea/Teepol solution: Solutions A. Equal volumes of water and Teepol CH-53 (Shell Malaysia Limited). Solution B. 16 g urea dissolved in 20 ml solution A; solution may be hastened by warming. Solution C. 20 g urea dissolved in 25 ml water. Solution D. 19 ml solution C + 1 ml solution A + 1 ml 12% w/v trichloroacetic acid. Solution E. 24 ml water + 1 ml solution A.

Eluting solution. 50 ml water + 50 ml acetone + 2 ml of 2-amino-2 methyl propan-1-ol (B.D.H. Ltd.).

Cellulose absorbent. Cellulose powder (10 g; Whatman, Standard Ashless Grade or Whatman CF11) is suspended in 100 ml of 1% w/v disodium EDTA and washed in the form of a column (approximately 2 cm diameter) with a further 200 ml 1% disodium EDTA followed by 200 ml water. The treated cellulose is washed by decantation several times with water to remove "fines"; and stored under water. It is kept in a refrigerator at 0–4° to reduce bacterial and fungal growth; any unused after 2 weeks is discarded.

Absorption columns. A column consists of a 50 mm length of 5 mm bore glass tubing; the lower end constricted down to a 1 mm hole and a wide bore reservoir section fixed to the upper end. The tube was constricted by heating the extreme tip in a fierce flame, this produced a flat topped ledge. On this ledge rests a 5 mm disc of

glass fibre filter paper (Whatman GF/A) which in turn supports the cellulose absorbent. Ordinary cellulose filter paper is not suitable since the resulting flow rate is too slow.

The columns are packed with a suspension of the treated cellulose in solution E. Care is taken to ensure a reasonable constancy of column height (27–28 mm.) The columns are allowed to drain and are then washed with 2 × 1 ml solution E. Shortly before the addition of the protein solutions they are washed with 1 ml solution B.

Method

Blood (0·5 ml) is added to 0·05 ml heparin solution (100 u/ml 0·9% NaCl) and centrifuged to separate the plasma. Plasma (0·25 ml) is transferred to a narrow 3 ml polypropylene tube (type 59400 M.S.E. Ltd.). Water (0·5 ml) is added followed by 12% trichloroacetic acid (0·5 ml) and the mixture shaken; after standing for 10 min the precipitate is separated by centrifuging at 1000 *g* for 5 min. The supernatant is poured off and its choline content assayed; the precipitate is drained for a few minutes by inverting the tube on filter paper and then 1 ml of solution B is added. The precipitate is brought into solution with the aid of a glass pestle. This consists of a piece of 4 mm glass tubing with a bulb blown at the end so that it fits the bottom and sides of the centrifuge tube closely. By gentle up and down strokes the precipitate is "homogenized" and dissolves. Frothing is prevented by lightly smearing the pestle shank with silicone antifoam A (Hopkin & Williams Ltd.). When the precipitate has dissoved, the pestle is withdrawn while being washed with a further 0·2 ml solution B. At this stage the solution may be left for some hours at room temperature.

The protein solution is passed through the prepared column to remove the azovan blue. Unaided, the flow is slow and the rate is best increased by the cautious application of compressed air at a pressure of about 20 cm water. The column must

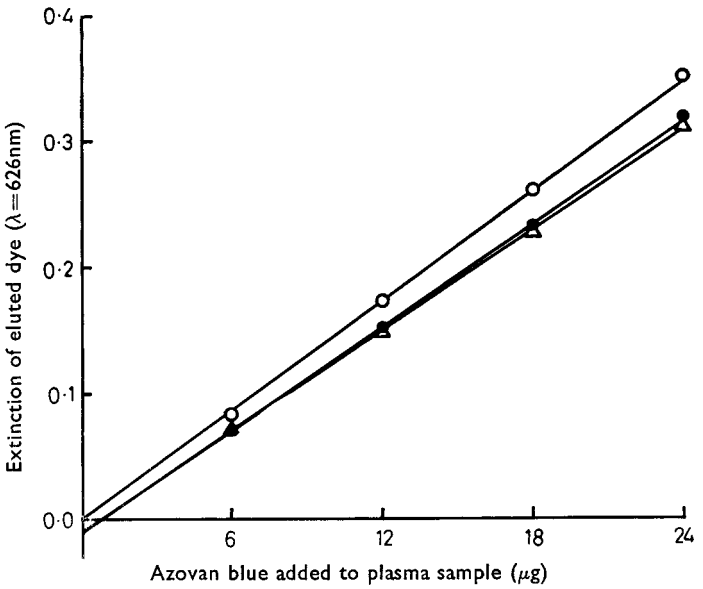

FIG. 1. Recovery of azovan blue added to rabbit plasma. ○ Original dye solution, ● Eluted from column after absorption from undenatured plasma and solution E (1 : 1), △ Eluted from column after absorption from precipitated and redissolved plasma proteins as described in method. Each point is the mean from duplicate samples. The lines shown are the calculated regression lines extended to the extinction axis.

be kept wet and too fast a flow will result in "break-through" of the dye. The centrifuge tube and column are washed free of protein with 2 × 1 ml solution D, each portion being allowed to pass through the column before the next is added. The column is washed with 2 × 1 ml solution E to remove most of the urea and the dye eluted by 3 × 1 ml of eluting solution. The dye solution is collected in 5 ml graduated stoppered test-tubes and made up to volume with eluting solution.

The extinction of the dye solution is read at 626 nm with eluting solution as blank. The relation between the extinction and the amount of dye in the plasma sample is linear from 0 to at least 30 μg of dye. The line for the recovered dye is almost parallel to that of the original dye solution but with a negative intercept on the extinction axis suggesting that there is a constant loss of dye independant of concentration (see Fig. 1). When 24 μg of dye is added to the plasma sample before the trichloroacetic acid precipitation, the recovery is 85–90%, this compares with 90–95% for the more usual procedure using undenatured plasma.

Standardization

At the start of an experiment four initial samples are taken before any dye is administered to the animal and known amounts of the injection solution added to the plasma just before the precipitation stage. Usually the amounts correspond to 6, 12, 18, 24 μg azovan blue and the regression line of extinction against amount of azovan blue is calculated by the method of Aldridge, Berry & Davies (1949). The dye concentration in the later samples is calculated from their extinction readings.

DISCUSSION

The method is a variant of many previously described for the extraction of azovan blue from plasma by the use of anionic detergents and cellulose. The use of cellulose treated with EDTA as absorbent and the addition of an organic amine to the eluting solution were adopted since they had been found to give higher recovery of dye from undenatured plasma. The nature of the amino-alcohol is probably not critical, ethanolamine would probably be satisfactory but none was available in the laboratory. Similarly the kind of Teepol used is a mixture of ionic and non-ionic detergents; Teepol 514 has been used on an earlier occasion, but it too is not obtainable in Singapore. Detergents other than Teepol CH-53 may require modification of the solutions, especially the amount of trichloroacetic acid in Solution D.

The plasma proteins can be brought into solution with strong urea solutions containing less Teepol but the absorption and recovery of the dye is not satisfactory.

The method although developed for the bioassay of choline can obviously be used with any other determination involving the removal of the plasma proteins by trichloroacetic acid. It has been used successfully with cat, rabbit, rat and guinea-pig plasma.

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