Chromatographic Determination of Evans Blue in Plasma and Serum

BY C. J. O. R. MORRIS (Freedom Research Fellow), *Clinical Laboratory and Medical Unit, the London Hospital*

(*Received* 11 *May* 1944)

The methods at present available for the determination of the dye, Evans blue, in plasma have certain disadvantages. Direct colorimetry of dyed plasma is inapplicable where the plasma is haemolyzed, turbid or lipaemic. The butanol extraction procedure of Harington, Pochin & Squire (1940) is troublesome and time-consuming where many analyses must be carried out; its accuracy is lower than those of other methods (Bowler, Crooke & Morris, 1944); it is liable to interference by haemolysis; and the modification suggested for haemolyzed plasma has not proved satisfactory in this laboratory. The method of Crooke & Morris (1942), which involves precipitation of plasma proteins and pigments with a hydrochloric acid-ethanolic phosphotungstic acid mixture, also has its disadvantages. It is likewise liable to interference by haemolysis, although this can be obviated by an optical correction method (Morris, 1944); and in certain specimens of phosphotungstic acid there is a substance which brings about the photochemical oxidation of Evans blue to a colourless compound. This difficulty can be overcome by working in artificial light. Certain plasma samples do not give an absolutely clear supernatant fluid on precipitation, and the occurrence of this effect, although uncommon, cannot be predicted. In Phillips's (1943) method the effect of interfering substances is allowed for by measuring residual light absorption after the Evans blue has been reduced to a colourless compound with sodium hydrosulphide. The method gives satisfactory results in the presence of a considerable degree of haemolysis, but the reduction requires 12 hr. for completion, and a supply of pure carbon monoxide is necessary.

In the course of an investigation of the homogeneity of commercial samples of Evans blue, it was observed that the dye was adsorbed from aqueous solution by aluminium hydroxide. This suggested that it might be possible to effect a quantitative separation of the dye from plasma or serum by chromatography. The adsorption behaviour of Evans blue in solution in plasma is, however, quite different from that of the dye in aqueous solution. Evans blue is only adsorbed from plasma at approximately pH 10. At this pH it is not adsorbed from aqueous solution. Ogston (1943) has shown by means of the ultracentrifuge that Evans blue combines with the plasma proteins, and it is therefore probable that the difference in adsorption behaviour, and also certain differences in light absorption (Phillips, 1943), are due to the existence of an Evans blue protein complex. With suitable conditions, however, it is possible to separate the dye from plasma by chromatographic means, the plasma proteins and pigments passing directly through the adsorption column. If the sample is haemolyzed, a portion of the alkaline haematin formed on adjusting to pH 10 is adsorbed on the column, but this can be removed by elution with acetic acid-ethanol. The dye is subsequently eluted from the adsorbent with hydrochloric acid-ethanol and the concentration in the eluate determined colorimetrically. This method permits a complete separation of Evans blue from all other light-absorbing substances present in plasma. If it is necessary to measure very low dye concentrations, it is possible to achieve a high degree of accuracy by concentrating the dye from larger plasma samples than those usually employed.

METHODS

Reagents

Adsorbent: 1 part (by wt.) of MgO Analar and 20 parts of $Al(OH)_3$ (The British Drug Houses, Ltd. 'dry') are thoroughly mixed by shaking in a bottle.

2 N-NaOH *solution*.

Acetic acid-ethanol wash liquid: 1 vol. glacial acetic acid Analar and 9 vol. 70 % (by vol.) ethanol.

Eluent: 8 vol. water, 10 vol. conc. HCl (sp. gr. 1·18) and 32 vol. absolute ethanol.

Apparatus

A two-holed rubber bung is provided with a sintered glass micro-filter tube (porosity 2, type 232 H2, Baird and Tatlock Ltd.) and a side tube for connexion to the water pump. 15 ml. centrifuge tubes are used as receivers.

Technique

Determination of Evans blue. The dry filter tube is filled with the adsorbent to a depth of about 2 cm. Uniform packing is ensured by tamping with a glass pestle during the filling. 1·00 ml. of dyed plasma or serum is measured into a suitable tube; 0·30 ml. of 2 N-NaOH is added and thoroughly mixed. 1·00 ml. of the mixture is pipetted on to the adsorption column and allowed to wet the adsorbent by capillary action. Gentle suction is now applied until the mixture has passed through the column. A bright blue zone forms on the upper part of the adsorbent. The column

is washed with 5 ml. of water which removes remaining plasma proteins and pigments, and with 4 ml. of the acetic acid-ethanol mixture which elutes any haem pigments present. The wash liquid is removed as completely as possible by suction, the receiver changed and the dye eluted with the HCl-ethanol mixture. The bright blue zone passes down the column and into the filtrate. About 8 ml. of eluent are necessary for complete elution. The eluate is transferred quantitatively to a 10 ml. volumetric flask and diluted to volume with the eluent mixture. The dye concentration is then determined colorimetrically.

Colour measurement

The determinations described in this work were made with a photoelectric absorptiometer constructed in this laboratory. An Ilford spectrum orange filter (no. 607, maximum transmission at 6000 A.) was used with a tungsten filament light source. Infra-red radiation was removed by a 2 mm. thickness of Chance calorex glass. Extinctions were measured in 20 mm. solution thickness. The dye concentration can then be found by reference to a standard curve. It is, however, advisable to check the concentration of the solution used for injection in plasma volume determinations by diluting 0·10 ml. of a 1 in 50 dilution of this injection solution to 10 ml. with eluent mixture and determining its extinction. This value can then be used as a standard in plasma volume determinations. All extinctions were measured against eluent mixture as control. The absence of interfering substances in the solutions to be measured, and the fact that solutions of Evans blue obey the Beer-Lambert law under the conditions used, suggests that it should be possible to obtain reliable results with a visual Duboscq pattern colorimeter. In this case larger plasma samples (2–3 ml.) should be used to avoid the difficulty of matching low colour intensities.

RESULTS

Light absorption of Evans blue in ethanolic HCl *solution*

The values given below were obtained by diluting known quantities of dye solution to 10 ml. with eluent mixture:

Evans blue (μg.)	Extinction
7	0·105
14	0·209
28	0·409
42	0·629

It will be seen that the Beer-Lambert law is obeyed.

Recovery of Evans blue from non-haemolyzed serum

Varying amounts of Evans blue were added to non-haemolyzed serum (1 ml.) and taken through the routine process. The values (corrected for volume differences) were as follows:

Evans blue present (μg.)	Evans blue found (μg.)	Recovery (%)
7·0	6·95	99·2
14·0	14·0	100·0
28·0	28·0	100·0
42·0	41·0	97·6

It will be seen that recovery is less complete at higher dye concentrations. These levels will, however, only rarely be reached even when two plasma volume determinations are carried out in rapid succession. With the amount of dye usually injected in the course of a plasma volume determination (35 mg.) the plasma concentration is 10–15 μg./ml.

Recovery of Evans blue from haemolyzed serum

14 μg. of Evans blue were added to 1·0 ml. of non-haemolyzed serum and kept for 10 min. Varying amounts of haemoglobin were added and the mixture taken through the routine process. The results are given below. It will be seen that the recovery is independent of the amount of haemoglobin present. Determination of the extinctions of the solutions with a combination of Ilford spectrum violet and Ilford aviol filters (Morris, 1944) indicated that the amount of haem pigment present was negligible. If the haemoglobin is added to the serum before the Evans blue, it apparently combines with the dye to some extent and the haemoglobin-Evans blue complex is removed by the acetic acid-ethanol wash liquid with consequent loss of dye. It is possible that the method will not be applicable to cases where there is considerable intravascular haemolysis.

Hb present (mg.)	Evans blue present (μg.)	Evans blue found (μg.)
1·5	14·0	14·0
3·0	14·0	14·0
4·5	14·0	14·0

SUMMARY

A new method for the determination of Evans blue is described, depending on the selective adsorption of the dye on a column of aluminium hydroxide and magnesium oxide, and subsequent elution with hydrochloric acid-ethanol. The dye concentration in the eluate is measured photometrically.

The expenses of this research were defrayed from the Yarrow Fund of the London Hospital.

REFERENCES

Bowler, R. G., Crooke, A. C. & Morris, C. J. O. R. [1944]. *J. Physiol.* (in the Press).

Crooke, A. C. & Morris, C. J. O. R. [1942]. *J. Physiol.* **101**, 217.

Harington, C. R., Pochin, E. E. & Squire, J. R. [1940]. *Clin. Sci.* **4**, 311.

Morris, C. J. O. R. [1944]. *J. Physiol.* **102**, 441.

Ogston, A. [1943]. Quoted by Courtice, F. C. [1943]. *J. Physiol.* **102**, 290.

Phillips, R. A. [1943]. *J. exp. Med.* **77**, 421.