THE EXTRACTION OF EVANS BLUE (T1824) FROM PLASMA AND THE MEASUREMENT OF PLASMA VOLUME

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(RECEIVED FOR PUBLICATION NOVEMBER 5, 1958)

Haemolysis and lipaemia interfere with the direct photometric estimation of Evans blue in plasma. During an investigation of blood volume changes in surgical shock using this dye, lipaemia was often encountered and it was necessary to use an extraction procedure for the estimation of the dye concentration. A rapid method was desired.

The method of Crooke and Morris (1942) using a hydrochloric acid - ethanol - phosphotungstic acid reagent is quicker to use than the chromatographic procedures described by Allen (1951) and by Bedwell, Patterson, and Swale (1955). Morris (1944b), however, described certain disadvantages, including that due to haemolysis, although Morris (1944a) had described an optical correction for the presence of haemoglobin in dyed plasma using filters with peak transmission at 600 $m\mu$ and 430 $m\mu$.

Because the original authors gave only a brief account of their experiments, the method was investigated in detail. The procedure for shaking and centrifuging was modified. Spectrophotometry at an optical depth of 4 cm. was used and a spectrophotometric correction for the presence of haemoglobin was introduced. On the basis of these experiments, which are detailed later, the following method was adopted, and was found to be free from the main disadvantages described by Morris (1944b).

Method for Measurement of Plasma Volume

Extraction Reagent.—Phosphotungstic acid (Hopkin and Williams, Analar, granular form), 7.5 g., is dissolved in 50 ml. of absolute alcohol. Then 8 ml. of concentrated hydrochloric acid (A.R.) is added slowly with shaking. The mixture is filtered through a fluted Whatman No. 1 filter paper. This reagent is best made up shortly before use, as a blue colour has been said to develop on exposure to light (Crooke and Morris, 1942).

Different preparations of phosphotungstic acid vary in their solubility in alcohol. The granular form

dissolves rapidly and the resulting solution has only a small light absorption at 620 $m\mu$. This was not the case using some other specimens of phosphotungstic acid which were coloured.

Procedure.—A sample of blood is withdrawn from a vein with minimal stasis. A solution of Evans blue (usually 20 mg. in 5 ml. for adults) is then injected through the same needle from a calibrated syringe. Ten minutes later a second sample of blood is withdrawn from a different vein. Dried heparin is a suitable anticoagulant. The plasma is separated and the concentration of dye in the second sample is determined by comparison with standards after extraction.

An initial dilution of the same sample of dye injected is made in distilled water for preparing standards. Two standards are made by adding 0.1 and 0.2 ml. of the solution to 1 ml. of the patient's undyed plasma. The resulting dye concentrations should straddle that in the second sample obtained from the patient. Into two other centrifuge tubes are delivered 1 ml. of the patient's undyed and dyed plasma respectively. Then 7 ml. of the extraction reagent is added to each tube, which is stoppered with a rubber bung and shaken vigorously by hand for 10 seconds, immediately after the addition of the extraction reagent. This breaks up the protein precipitate, which otherwise sticks to the side of the tube resulting in incomplete extraction of the dye. All tubes are then shaken horizontally in the direction of the long axis in a Kahn shaker for 10 minutes. They are then capped and centrifuged for 10 minutes at 2,500 r.p.m. The temperature in the centrifuge bucket should not rise more than 5° C., otherwise turbidities develop (see below). The supernatant fluid is removed and the test and standards are read against the blank in a "unicam" spectrophotometer SP 500 or SP 600 at 620 $m\mu$ and 400 $m\mu$. The readings are made at an optical depth of 4 cm. using a modified cuvette holder to allow the use of a volume of 6 ml. At 620 $m\mu$ light absorption by Evans blue is maximal, and at this wavelength the dye obeys Beer's law in the extraction reagent over a range of concentrations of 0.1 to 0.4 mg./100 ml. This is a wider range than is encountered in plasma volume estimations after

the injection of 10 to 20 mg. of dye in adults. The optical density of extracted undyed plasma is very low at this wavelength. In order to correct for haemolysis in the second dyed sample, the optical density at 400 $m\mu$ is divided by an experimentally determined factor of 35 (see below). The value so obtained is subtracted from the optical density at 620 $m\mu$. If the test solution at 400 $m\mu$ yields a negative reading, this is due to greater haemolysis in the blank undyed plasma. In this circumstance the optical density of the blank solution is read against the test solution at 400 $m\mu$. This optical density is divided by 35 and added to that of the test solution at 620 $m\mu$. This corrects for haemolysis in the undyed plasma.

Calculation.—Let T be the optical density of the test solution corrected for haemolysis. If V be the volume of dye injected, and S_1 and S_2 the optical densities of the standards, and if the initial dilution of the dye in water is 1 in 50, then plasma volume

$$= \frac{S_1}{T} \times V \times \frac{50}{0.1} \times \frac{8.1}{8.0} \text{ ml.}$$
$$\text{or} = \frac{S_2}{T} \times V \times \frac{50}{0.2} \times \frac{8.2}{8.0} \text{ ml.}$$

Experimental Results

Unless otherwise stated, the procedure was that already described under "Method."

Duration of Shaking Required for Maximum Extraction.—Quantities of plasma, each of 1 ml., were added to a series of round-bottomed centrifuge tubes. To some of these exactly 0.1 ml. of a solution of Evans blue was added. Then 7 ml. of extraction reagent was added to all tubes, which were stoppered and shaken. Tubes containing dye were removed in pairs after varying intervals of time together with one of the tubes

FIG. 1.—The effect of duration of shaking on the extraction of Evans blue.

containing undyed plasma. Using the latter as a blank the optical density of the dye solution was determined. Fig. 1 shows the variation of extraction with time. Extraction was maximal after eight minutes; the degree of extraction after shaking for five minutes was only very slightly less.

Consistency of Extraction.—Replicate extractions were made of three different solutions of Evans blue in plasma. The results are shown in Table I. The mean optical densities of the extracted solutions were 0.613, 0.318, and 0.169.

TABLE I
CONSISTENCY OF EXTRACTION OF T1824 FROM PLASMA

Experiment	No. of Replicates	Mean Optical Density	Standard Deviation	Coefficient of Variation (%)
1	7	0.613	± 0.004	0.7
2	16	0.318	± 0.003	1.0
3	7	0.169	± 0.003	1.8

The standard deviations of the means were 0.004, 0.003, and 0.003 respectively. In plasma volume estimations the optical densities encountered fall within the range of 0.100 to 0.400 if doses of 12 to 20 mg. of dye are used in adults.

Efficiency of Extraction.—The extraction of a series of dye dilutions in plasma was compared with a series in which the extraction reagent was added to the same dilutions of dye in distilled water. All extractions were in duplicate, and the results are shown in Table II. The degree of

TABLE II
EFFICIENCY OF EXTRACTION OF T1824 FROM PLASMA COMPARED WITH WATER

Approximate Concentration of Dye (mg./100 ml. Final Solution)	Optical Density ($m\mu$)				% Extraction
	Plasma Duplicates	Water Duplicates	Mean of Plasma Duplicates	Mean of Water Duplicates	
0.03	0.077 0.078	0.078 0.076	0.0775	0.077	101
0.06	0.166 0.165	0.167 0.169	0.1655	0.168	98.5
0.10	0.302 0.306	0.308 0.312	0.304	0.310	98
0.10	0.312 0.312	0.319 0.319	0.312	0.319	98
0.12	0.330 0.328	0.341 0.342	0.329	0.3415	96
0.16	0.446 0.450	0.462 0.460	0.448	0.461	97
0.20	0.611 0.608	0.622 0.627	0.6095	0.6245	97.5

extraction was 96%, or more, in all cases. No allowance is made for the space occupied by protein, since this would affect the results by less than 1%.

A similar comparison was made with standard solutions in plasma and distilled water during routine plasma volume estimations. In 15 consecutive estimations the mean percentage extraction was 97.6 (standard deviation ± 1.4).

Correction for Haemoglobin in Either Dyed or Undyed Plasma.—Haemoglobin is extracted as acid haematin, which has an absorption peak at 400 $m\mu$, at which wavelength light absorption by Evans blue (T1824) is small (Fig. 2).

FIG. 2.—The spectral absorption of Evans blue and acid haematin in the extraction reagent.

When 7 ml. of extraction reagent was added to 1 ml. of solutions of haemoglobin in plasma it was found that the resulting acid haematin solutions obeyed the Beer-Lambert law at 400 $m\mu$ and 620 $m\mu$ for original plasma haemoglobin concentrations of less than 100 mg./100 ml. Above this concentration there was a departure from the straight line relationship. One hundred milligrams haemoglobin/100 ml. plasma represents considerable haemolysis. In over 400 estimations of plasma volume, plasma haemoglobin concentrations were less than 30 mg./100 ml. in all except one case.

A factor for correcting for the presence of haemoglobin was obtained by determining the ratio $\frac{\text{optical density at } 400 \text{ } m\mu}{\text{optical density at } 620 \text{ } m\mu}$ for different plasma haemoglobin concentrations as follows.

A large volume of undyed plasma was extracted in the usual manner. To 8 ml. aliquots of the supernatant fluid was added 0.1 ml. of different haemoglobin solutions. The tubes were shaken and centrifuged, and the optical density measured at both wavelengths using a blank consisting of 8 ml. of supernatant fluid and 0.1 ml. of distilled water. The optical depth was 4 cm. at 620 $m\mu$ and 1 cm. at 400 $m\mu$. The densities at 620 $m\mu$ were therefore divided by 4 when calculating the ratio.

In a similar manner 0.1 ml. quantities of haemoglobin were added to 1 ml. of plasma and extracted as before. The results obtained in these two experiments were substantially the same, and those obtained in the second experiment are shown in Table III.

It is seen that the ratio varies from 28 to 38, the mean value being 35. The largest variations from the mean occur with the lowest haemoglobin concentrations, when small absolute errors have relatively large effects on the ratio, but where the correction for haemoglobin is very small.

In the above experiments, if the observed optical density at 400 $m\mu$ is divided by the ratio, the value obtained represents the optical density due to acid haematin at 620 $m\mu$. If the mean ratio of 35 is used for this calculation, the results agree closely with the observed values at 620 $m\mu$. Even in those cases where the experimentally determined ratio differs greatly from the mean, the value obtained by dividing by 35 differs from the observed optical density at 620 $m\mu$ by less than 0.002, which is the precision of the photometric readings.

Correction for haemolysis during Evans blue estimation may therefore be made in the manner previously described in the procedure for measurement of plasma volume. For the greatest accuracy, allowance should be made for the absorption due to Evans blue at 400 $m\mu$. This value is very small, however, and ignoring it results in an error in the

TABLE III
DETERMINATION OF THE RATIO $\frac{\text{OPTICAL DENSITY AT } 400 \text{ } m\mu}{\text{OPTICAL DENSITY AT } 620 \text{ } m\mu}$ FOR Hb IN PLASMA

Hb concentration (mg./100 ml.)	9.6	19.2	38.4	45	76.8	115	144	153.6	180	192
Density, 400 $m\mu$, 1 cm. . .	0.064	0.135	0.258	0.320	0.524	0.778	0.957	1.019	1.178	1.239
„ 620 „ 1 „ . . .	0.0023	0.0045	0.00775	0.0085	0.0152	0.0215	0.0278	0.0298	0.0335	0.0367
Ratio D400/D620 . . .	28	30	33	38	35	36	35	34	35	34

corrected optical density at 620 $m\mu$ of less than 0.001.

The accuracy of this correction factor is shown in Table IV. Replicate extractions were made of 1 ml. aliquots of dyed plasma. Three of the tubes also contained different amounts of haemoglobin and two others contained distilled water so that the final volume was the same in all tubes. The optical densities were all determined against the same plasma blank. After correction, the haemolysed samples gave a value for Evans blue which is virtually identical with unhaemolysed samples.

TABLE IV
CORRECTION FOR PRESENCE OF HAEMOGLOBIN IN DYED PLASMA

Tube:	1	2	3	4	5
Haemoglobin solution (ml.)	0	0	0.1	0.1	0.1
Distilled water (ml.)	0.1	0.1	0	0	0
Observed optical density at 620 $m\mu$	0.118	0.115	0.124	0.136	0.151
Corrected optical density at 620 $m\mu$	0.118	0.115	0.118	0.117	0.115

Turbidity in Plasma Extracts.—In earlier experiments clear extracts were always obtained even when the filtered extraction reagent was not absolutely clear. In later experiments solutions frequently became turbid after centrifugation. This was found to be due to a rise in temperature of the centrifuge. Extracts always became turbid on standing if the temperature of the centrifuge bucket rose more than 5° C. above room temperature. This did not occur if the temperature in the centrifuge bucket did not rise or if the tubes were cooled in ice for five minutes before centrifuging.

Reproducibility of the Method *in vivo*.—The plasma volume was measured twice in 10 subjects with an interval of 10 minutes between the two measurements. The subjects were healthy students and members of the laboratory staff. All estimations were made in the laboratory with the subjects sitting for the duration of the test.

A sample of blood was withdrawn and 5 ml. of a 0.25% or 0.4% solution of Evans blue was injected through the same needle. Ten minutes later a second sample of blood was withdrawn from a different vein and a second dose of dye injected through the same needle. A third sample of blood was withdrawn from another vein 10 minutes later. The plasma volumes were obtained from the concentrations of dye in the second and third samples described above. The haematocrit was determined on each specimen to assess changes during the test. No significant change occurred.

The results are shown in Table V. It is seen that the difference between the two estimations is less than 2.5% in all cases. In seven cases the second estimation is greater than the first. This difference is significant ($t=2.711$; $P<0.05$), and is due to the fact that the second specimen is used as a blank for the second estimation.

TABLE V
REPRODUCIBILITY OF PLASMA VOLUME ESTIMATION IN NORMAL SUBJECTS

Subject	Plasma Volume 1 (ml.)	Plasma Volume 2 (ml.)	Difference (ml.)
1	2,430	2,460	+30
2	3,900	3,960	+60
3	2,920	2,880	-40
4	2,860	2,890	+30
5	3,730	3,780	+50
6	3,770	3,780	+10
7	3,980	3,050	+70
8	2,960	2,960	0
9	3,500	3,510	+10
10	2,870	2,900	+30

Approximately 1.5% of the dye first injected leaves the circulation in the second 10 minutes (Gibson and Evans, 1937). This leads to a false low reading for the dye concentration in the third specimen and hence a slight overestimate of plasma volume.

Discussion

Crooke and Morris (1942) claimed that extraction of dye was 100%, but reported the results of only one experiment. Reeve (Grant and Reeve, 1951) obtained over 90% extraction in 70 out of 75 estimations. We found in early experiments that extraction was often irregular due to precipitated protein sticking to the wall of the centrifuge tube. Since adopting the practice of shaking the tubes vigorously by hand immediately after the addition of the extraction reagent, extraction has been consistently greater than 95%. This compares well with the value of 97% achieved by Allen (1951) and by Bedwell *et al.* (1955), using chromatographic techniques. Extraction is consistent over a wide range of concentrations. Errors due to incomplete extraction are minimized by the use of standards prepared in the manner described. Certain photochemical effects were noticed by Crooke and Morris (1942) and Morris (1944b). The extraction reagent was found to turn blue after exposure to daylight for 48 hours, and certain batches of phosphotungstic acid were found to decolorize Evans blue. Reeve (Grant and Reeve, 1951) carried out extractions with very little exposure to light and encountered neither of these effects. This has also been our experience. Exposure to

direct sunlight has been avoided and the extraction reagent has been used within 12 hours of preparation. The optical densities of the standards have shown very little variation in several hundred plasma volume estimations, so that decolorization is most unlikely.

Morris (1944b) stated that occasional plasma samples did not yield an absolutely clear supernatant fluid after precipitation. This was uncommon but unpredictable. Grant and Reeve (1951) found that careful handling and sufficient centrifuging always resulted in clear solutions. In the present investigation clear extracts were always obtained if the temperature in the centrifuge bucket did not rise during the centrifuging. It appears that at temperatures above about 20° C. some substance dissolves in the reagent and precipitates on cooling.

The optical correction for the error due to haemolysis described by Morris (1944a) yielded comparable values for extinction due to Evans blue, if the dye was added to extracts of plasma containing varying amounts of haemoglobin. However, the presence of haemolysis has been considered a disadvantage of this method (Morris, 1944b). The spectrophotometric correction described here is simple and gives good results.

The method has been found to be unsatisfactory in a few cases with plasma bilirubin concentrations of 10 to 20 mg./100 ml. Alteration in the nature of this pigment results in a second absorption peak in the red end of the spectrum so that high blank values are obtained. The optical densities of

duplicate extractions of deeply jaundiced plasma may sometimes differ widely.

Summary

The extraction of Evans blue from plasma using the hydrochloric - acid - ethanol - phosphotungstic acid reagent of Crooke and Morris has been investigated.

Minor modifications of the procedure for shaking and centrifuging are described. The optical density of the extracted solutions is determined spectrophotometrically using an optical depth of 4 cm. A simple spectrophotometric correction for the presence of haemolysis is described.

With the technique described extraction is consistent and greater than 95%, and turbidity of the extracted solutions is avoided.

Duplicate plasma volume estimations agree within 2.5%.

Various criticisms of the method are discussed.

We wish to thank Dr. G. K. McGowan for his advice and criticism during this work and for his help in the preparation of this paper. We thank Mr. E. Lloyd for preparing the solutions of Evans blue.

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