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Note

A method of assay for sulphobromophthalein and other dyes in whole blood by a DEAE-cellulose column technique

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The use of DEAE-cellulose column chromatography for the assay of sulphobromophthalein (BSP), bromphenol blue (BPB) and azo dyes in whole blood has been investigated in the past. Although it has been very common to determine BSP in plasma colorimetrically after treatment with sodium hydroxide, this simple method has two disadvantages. Firstly, even slight haemolysis, bilirubin or turbidity, usually due to lipoproteins and lipids, cause interference in the determination. Secondly, in order to study the time course of the concentration of BSP in whole blood in small animals, too large an amount of blood is required. Therefore, these two weak features of the method have not made it possible to investigate accurately the biotransformation of these drugs, particularly in small animals. Various methods have been proposed for eliminating the above interferences. Gaebler[1] and Reinhold[2] made corrections for these factors by reading optical densities at two or three wavelengths. Seligson *et al.*[3] proposed a method which eliminated errors due to protein binding with albumin by treatment of serum with sodium *p*-toluenesulphonate. However, these methods do not alter the requirement for a large volume of blood.

In this paper, a simple and sensitive assay method with high accuracy for BSP, BPB and azo dyes in rat whole blood using a DEAE-cellulose (HCO_3^-) column is reported, in which the two previous difficulties are overcome.

EXPERIMENTAL

Materials

DEAE-cellulose (Brown, New York, N.Y., U.S.A.: 0.90 mequiv./g) was used after conversion into the OH^- form by a known method[4]. BSP was obtained from K & K Labs. (Hollywood Calif., U.S.A.). BPB, azorubin S (AS), amaranth (AM) and new coccine (NC) were obtained from Tokyo Chemical Industries (Tokyo, Japan).

Preparation of column

DEAE-cellulose (OH^-) was packed to a height of about 4 cm in a glass column

(1.2 × 15 cm). It was then converted into the HCO_3^- form with 20 ml of 1 *M* $KHCO_3$, and washed with water until OH^- and HCO_3^- were not eluted.

Procedure for assay of BSP in whole blood

Rat whole blood (0.05–0.10 ml) containing BSP (5–200 μg) was haemolyzed by the addition of an aliquot amount of water and applied to the top of the prepared column. The sample that remained on the column wall was washed down with at least two successive 5-ml portions of water, and an additional cellulose (HCO_3^-) layer prepared in advance was then placed on top of the column to a height of about 0.5 cm. The column was washed through with 50–100 ml of water followed by 20 ml of 0.25 *M* potassium hydrogen carbonate solution in order to eliminate the blood components. After an additional wash with about 20 ml of water, the dye was eluted with 20 ml of 0.05 *N* sodium hydroxide solution and the alkaline eluate collected in a 25-ml measuring flask. After the mixture in the flask had been made up to the calibration mark with water, the absorbance of the dye solution was measured at 578 nm against a blank. The blank was prepared from a control blood (obtained before the infusion of dye) taken through the same procedure.

RESULTS AND DISCUSSION

As BSP has a high affinity for the blood proteins, it has previously been impossible to determine it by a deproteinizing procedure. In the present study, the use of a weak basic anion exchanger, DEAE-cellulose (HCO_3^-), used extensively for separating proteins, has made it possible to separate completely the dye from the blood components. Table I shows the relationship between absorption and concentration of dye obtained from the assay of BSP (5–100 μg) in rat whole blood (0.08 ml).

TABLE I

ASSAY OF BSP IN RAT WHOLE BLOOD

BSP (μg)	*Absorbance (578 nm)*
5	0.016
10	0.032
20	0.061
40	0.125 ± 0.001 (14)*
60	0.188
80	0.258 ± 0.003 (4)*
100	0.315

* Mean ± S.D. in the number of experiments given in parentheses.

Conformity with Beer's law over the entire concentration range was obtained. The recovery of BSP (5–100 μg) added to the blood (0.05–0.10 ml) was 94–100%, which varied slightly according to volume of blood used. It was observed that the use of a higher volume of blood gave a lower recovery. In a practical experiment, a correction can be made by running through an aliquot of control blood containing a standard dye solution.

BPB has been used as a model compound of anionic drugs in a study of trans-

port mechanisms in the liver, and its assay in whole blood can be carried out in a similar manner to that for BSP. A 25-ml volume of 0.20 *M* potassium hydrogen carbonate solution was used instead of the 0.25 *M* solution in order to wash out the blood components. The absorbance of the alkaline eluate was measured at 590 nm. As shown in Table II, conformity with Beer's law over the entire concentration range from 5 to 160 μg was obtained. The recovery of BPB (5–160 μg) added to the blood (0.05–0.10 ml) was 98–100%.

TABLE II

ASSAY OF BPB IN RAT WHOLE BLOOD

BPB (μg)	*Absorbance (590 nm)*
5	0.021
10	0.042
40	0.162
80	0.331 ± 0.004 (3)*
120	0.497
160	0.667 ± 0.003 (3)*

* Mean ± S.D. in the number of experiments given in parentheses.

The azo dyes (AS, AM and NC) have also been used as model compounds in a study of transport mechanisms in the liver[5]. Assays of these azo dyes in whole blood can also be carried out in a similar manner to that for BSP. In order to elute these dyes, 20 ml of 0.1 *N* sodium hydroxide solution was used instead of the 0.05 *N* solution, and the eluate was neutralized with 2 ml of 1 *M* sodium dihydrogen orthophosphate solution. The mixture in the flask was made up to the calibration mark with water and the absorbance of the dye solution measured at 515 nm for AS, 522 nm for AM and 507 nm for NC. The results in Table III are given as an example of the determination of NC. Conformity with Beer's law over the entire concentration range from 5 to 100 μg was obtained. For AS and AM, similar good results were obtained. The recoveries of azo dyes (5–100 μg) added to the blood (0.05–0.10 ml) were 97–100% for NC, 92–100% for AS and 98–100% for AM.

The procedures for the assays for the five anionic dyes used are summarized in Table IV.

TABLE III

ASSAY OF NC IN RAT WHOLE BLOOD

NC (μg)	*Absorbance (507 nm)*
5	0.007
10	0.015
25	0.038
50	0.072 ± 0.001 (4)*
75	0.110
100	0.141 ± 0.004 (7)*

* Mean ± S.D. in the number of experiments given in parentheses.

TABLE IV

PROCEDURES IN ASSAYS OF DYES IN RAT WHOLE BLOOD

The alkaline eluate of each dye was collected in a 25-ml measuring flask, in which 1 *M* NaH_2PO_4 was placed for the azo dyes, and the flask was finally filled to the mark with water.

Dye	*Number of sulphonic acid groups*	*$KHCO_3$ concentration*		*NaOH concentration*		*Volume of 1 M NaH_2PO_4 for neutralization (ml)*	*Absorption maximum (nm)*
		(M)	*Volume (ml)*	*(N)*	*Volume (ml)*		
BSP	2	0.25	20	0.05	20	--	578
BPB	1	0.20	25	0.05	20	—	590
NC	3	0.25	20	0.10	20	2	507
AM	3	0.25	20	0.10	20	2	522
AS	2	0.25	20	0.10	20	2	515

It is obvious that the dyes with a larger number of sulphonic acid groups require a higher concentration of sodium hydroxide solution for elution. When two dyes are present in blood together (for example, BSP and BPB, BSP and azo dyes, and BPB and azo dyes), a differential assay may be possible by selecting the concentrations of potassium hydrogen carbonate solution used to wash out the protein and alkaline solution to elute the dye.

Although the assay for BSP in whole blood using deproteinizing reagents has not been possible because of its strong adsorption on the proteins, the use of a DEAE-cellulose column technique has made possible the assay of the dye in whole blood.

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