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THE ESTIMATION OF 5-HYDROXYTRYPTAMINE IN HUMAN BLOOD

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Abstract—A rapid and quantitative method for the assay of 5-hydroxytryptamine in whole blood is described. It is based on the ability of carboxymethylcellulose to bind the free amine in lysed blood in the presence of protein. The bound 5-hydroxytryptamine is eluted from a carboxymethylcellulose column and estimated by spectrophotofluorimetry. Fifty normal patients gave an observed range of 0·037–0·280 μg of 5-hydroxytryptamine/ml of whole blood.

MANY methods, both biological and biochemical have been published for the estimation of 5-hydroxytryptamine (5HT) in tissues and body fluids; the subject has recently been reviewed by Sandler.[1] Although some of these are concerned with the estimation of 5HT in whole blood,[2–4] they tend to be specialised and time consuming. The procedure described here is simple and quick and can be used for the simultaneous assay of a large number of samples.

MATERIALS

Glassware was soaked in concentrated nitric acid for 16 hr before use. Chromic acid was not used for cleaning as it interfered with subsequent fluorimetric analysis.

EDTA Disodium salt of ethylenediamine tetra-acetic acid (Hopkin and Williams Ltd.). L(+) Ascorbic acid (Hopkin and Williams Ltd.). 5-Hydroxytryptamine creatinine sulphate (Roche Products Ltd.)

5HT is expressed as the free amine. Carboxymethyl cellulose CM 70 (Light and Co. Ltd. (CMC).

CMC (100 g) was stirred for ½ hr with 1½ l. 0·1 N HCl. Fines were removed from the slurry by its repeated suspension in distilled water followed by decanting the supernatant layer. The final product, tested for the absence of chloride ions, was suspended in Tris-HCl buffer (0·05 M, pH 8·0), titrated to pH 8 with 2 M Tris solution and stored in the wet state at 4°. Before use, the suspension was stirred for 15 min by a magnetic stirrer which released any air bubbles clinging to the CMC particles.

METHODS

Preparation of column

A glass column (internal dia. 1 cm, height 35 cm), with a thistle-funnel end to act as a reservoir, was filled with distilled water and a small pledget of cotton wool was used to plug the base of the column which was sealed with a clipped rubber tube. A slurry of stirred CMC powder was introduced under the surface of the water in the column by means of a wide-bore Pasteur pipette, making sure that air bubbles were

excluded. The CMC was allowed to settle by gravity flow on release of the rubber clip. The column was packed to the desired height, and a slight air pressure using a rubber bulb, was applied after the completion of the packing, to even out the top of the CMC column and to make it compact .Using this technique, the rubber clip was no longer necessary as the level of liquid was retained at the top of the CMC column without air being introduced into it and the column running dry.

Standardisation of the CMC

Batches of CMC were found to vary in their capacity for binding 5HT in the presence of other cations. The optimum height of a CMC column necessary to retain the maximum amount of 5HT that might be present in 2 ml of whole blood was determined as follows: 4 μg of 5HT were added to 2 ml of lysed blood, and the total 5HT determined by the procedure to be described, on CMC columns with heights of 2·5, 5, 7·5, 10, 12·5 and 15 cm. The column with the minimum height of CMC giving a maximum recovery of 5HT was selected for the assay procedure (see Table 1).

TABLE 1. OPTIMUM HEIGHT OF COLUMN FOR 2 ml OF BLOOD

Height of column (cm)	2·5	5·0	7·5	10	12·5	15·0
Total 5HT in eluates expressed as R.F.I.	110	223	509	554	543	485

R.F.I. = Relative Fluorescence Intensity

Treatment of the blood sample for the estimation of 5HT

Venous blood (5 ml) was obtained using a siliconed needle and a siliconed glass syringe, or a disposable needle and syringe, care being taken to avoid haemostasis. The blood was transferred to a plastic vial containing 10 mg EDTA and 15 mg ascorbic acid and gently mixed by several inversions. Using a siliconed pipette, 2 ml blood was added drop by drop to a centrifuge tube containing 30 ml buffer and a trace of octyl alcohol, through which a stream of coal gas (carbon monoxide content 17·6 $\pm$ 1%) was bubbled for 1–2 min. The centrifuge tube was sealed with 'Parafilm' and after centrifugation at 3000 rev/min for 15 min, the supernatant was transferred to the CMC column without disturbing its surface. The cell debris remaining in the tube was suspended in 20 ml of buffer, centrifuged for 10 min, and the supernatant transferred as before. The column was finally washed with 20 ml buffer followed by 7 ml 0·2 N HCl. This volume of HCl was necessary to displace buffer from the column before the actual elution of the 5HT started, at acid pH. The final elution of the 5HT was now done as follows. Any drop adhering to the tip of the column was removed. One ml 0·2 N HCl was pipetted on to the top of the column, and the eluate was collected in a small glass test-tube making sure that the last drop of eluate adhering to the tip of the column was included, to give a final vol. of 1 ml. Six such 1-ml portions of eluate were collected in separate test-tubes, and to each of these tubes was added 0·5 ml of 10 N HCl. The relative fluorescence intensity of the contents of each of these tubes was determined in an Aminco–Bowman Spectrophotofluorimeter, set for activation at 297 mμ and fluorescence at 535 mμ. One drop of H_2O_2 (10 vol.) was then added to each tube and a further reading was taken after mixing. This 'blank' reading

was subtracted from the first reading for each tube and the value of 5HT read from a standard curve, corrected for the volume, and the sum of the values obtained for each tube was expressed as μg 5HT/ml of whole blood. All estimations were carried out in duplicate. With the activation wavelength set at 297 mμ the fluorescence spectrum was scanned to confirm the characteristic peak of 5HT at 535 mμ. A typical scan is shown in Fig. 1, which also demonstrates the disappearance of the peak at 535 mμ after the addition of H_2O_2.

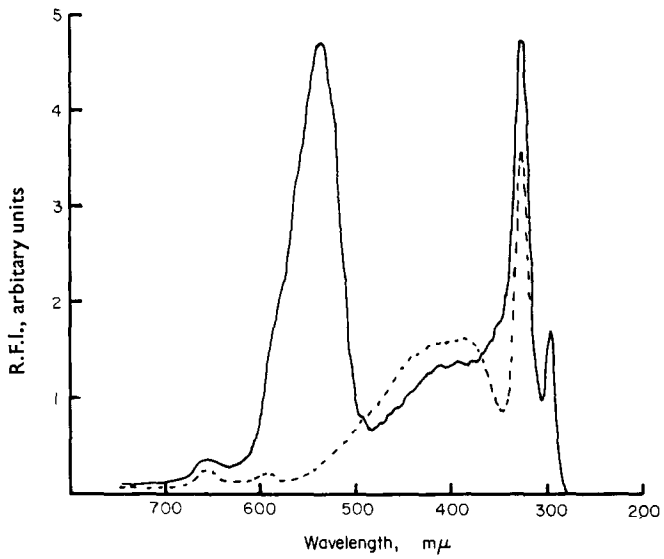

FIG. 1. The fluorescence spectrum of 5HT in 3 N HCl activated at 297 mμ before (———) and after (- - - -) the addition of H_2O_2.

Before the actual readings were taken, the fluorimeter was standardised to a given setting by means of the sensitivity control, using a known quinine standard (activation at 355 mμ and fluorescence at 445 mμ).

The instrument characteristics used were:
Slit arrangement: No. 3.
Lamp: XBO-150 W.
Detector: 1P 21 photomultiplier tube.

Platelets in blood

The platelets in each sample of blood were estimated using the Formol-Citrate method.[5]

METHOD

Standardisation of the CMC *column*

The 10-cm column was the minimum height found necessary for optimal recovery of 4 μg of 5HT added to 2 ml of blood.

Siliconing of apparatus

There was a 20–30% increase in levels of 5HT when syringes, needles and blood containers were siliconed compared with un-siliconed apparatus.

The use of disposable syringes and needles, with plastic blood-containers, gave identical results to those obtained with siliconed apparatus.

Stability of 5HT *in blood and its release from platelets*

There was no appreciable difference in 5HT levels between samples of the same blood containing EDTA alone or with ascorbic acid added, if the estimation was carried out within 24 hr of blood collection. The presence of ascorbic acid gave slightly higher recoveries after storage for 48 hr. Samples were stored at 4° when necessary.

A platelet count on the residue after the removal of the second supernatant buffer solution, showed platelets to be completely lysed. Levels of 5HT in replicates of 4 on the same blood sample, varied by less than ±5% of the mean value. This finding confirmed a consistent release of 5HT from platelets.

Effect of carbon monoxide

Saturation of lysed blood by coal gas produced a 30–40% increase in blood 5HT levels, although such saturation before lysis, caused 5HT levels to fall.

TABLE 2. RECOVERIES OF 5HT ADDED TO BLOOD

		Subjects			
		PE		CR	
	Samples	Total 5HT present (μg)	Recovery (%)	Total 5HT present (μg)	Recovery (%)
a	Blood + 5HT	0·310	98	0·528	100
b	Lysed Blood + 5HT	0·290	92	0·543	103
c	5HT	0·060	—	0·304	—
d	Blood	0·256	—	0·222	—

The percentage recovery of 5HT is calculated from the total amount of 5HT present in the blood. As the added 5HT in the case of subject PE is small compared to the 5HT present in the blood, a small error in the estimation of blood + 5HT would be considerably magnified if expressed as percentage recovery of added 5HT.

Optimum pH *and molarity of the buffer*

A number of different pH values between 7·0 and 8·2 were tested for maximum recovery of 5HT from blood; pH 8·0 was found to be optimal.

Thirty ml 0·05 M buffer was sufficiently hypotonic to lyse 2 ml blood while maintaining its pH at 8·0.

RESULTS

Blood 5HT *levels*

Fifty normal patients gave an observed range of 0·037–0·280 μg of 5HT/ml of whole blood. (Mean ± S.D. = 0·147 μg ± 0·057 μg).

Recoveries of 5HT *added to blood*

A solution of 5HT (0·2 ml in 0·001 N HCl) was added to (a) 2 ml of blood (b) 2 ml of blood lysed with 30 ml of buffer (c) 30 ml of buffer containing EDTA and ascorbic acid (equivalent to the amounts used for 2 ml of blood). The total amount of 5HT in (a), (b), (c) and in 2 ml of blood (without added 5HT), was estimated. The recoveries were calculated based on the recovery of 5HT from (c) (see Table 2).

The relationship between 5HT *and blood platelets*

Figure 2 shows the correlation between 5HT and blood platelets.

A linear regression was calculated and the correlation coefficient was found to be 0·32 with 48 degrees of freedom which is significant at the 5% level.

$y = 30{\cdot}2 + 0{\cdot}71x$.

DISCUSSION

Present methods for the estimation of 5HT in whole blood rely on initial precipitation of proteins followed by alkaline extraction,[4, 6] acid extraction,[7] or column absorption[2] procedures.

A weakly acidic cation exchanger such as Amberlite CG 50 resin has been used for the estimation of 5HT[8] and a modification of this procedure has been applied to its assay in blood.[9] There are disadvantages however in using ion exchange resin for blood 5HT measurement: precipitation of the proteins becomes a necessary step, and the preparation of the resin in its final form is a long and tedious operation, for unless exhaustive recycling and washing takes place, a very high resin 'blank' is obtained during subsequent fluorimetric assay. As CMC 70 acts as a weakly acidic cation exchanger and the presence of protein does not appear to affect its affinity for free 5HT, it was found to be very suitable for binding the 5HT followed by its subsequent elution. It is not surprising to find that the optimum pH for binding 5HT to CMC is 8, as it is at this pH that CMC is fully ionised. Other advantages to the use of this material are, that it requires very little initial preparation has no appreciable blank when used in fluorimetric assay procedures, is easy to pack into a column and cheap enough to be discarded after use.

The normal range of 5HT detected in blood by the present method (0·037–0·280 μg/ml) compares with the range found in normal, non-pregnant women (0·05–0·18 μg/ml) by Carter *et al*[9] using a weakly acidic cation exchanger. When the method was in its early stages of development, blood 5HT values on three patients with carcinoid syndrome were found to be roughly ten times that of the then normal values.

Following a report by Stacey,[10] Blum and Ling[11] have shown that 5HT is oxidised during the denaturation of oxyhaemoglobin. Rodnight[12] has shown this reaction to be inhibited by carbon monoxide, an observation which is confirmed here by the finding of an increase of 30–40% in 5HT values when coal gas, a suitable substitute for carbon monoxide was bubbled through lysed blood; frothing, which probably contributed to low 5HT values obtained from blood 'mixed' with EDTA and ascorbic acid by vigorous shaking, was prevented by adding a trace of octyl alcohol. Thorough mixing was assured however by inverting the vial gently several times. If clotting occurred, blood was discarded as it led to erratic values.

Boullin[13] has made an important contribution to the spectofluorimetric assay of 5HT. The 'blank' in such assays can contribute an appreciable error to the final

5HT value. The true level of 5HT can only be assessed by a reading which excludes non-specific fluorescence occurring at 535 mμ. This author has shown that the specific fluorescence of 5HT at low concentrations (1 μg/ml or less) disappears at the end of 6 hr, whereas non-specific fluorescence persists for much longer. He therefore advocated taking further readings at 1, 2, and 4 hr after the initial reading, scanning the fluorescence curve, and subtracting the blank reading from the initial reading when the peak at 535 mμ due to 5HT had disappeared. This is a tedious procedure and since the mechanism by which the specific fluorescence is removed is presumably oxidation, the process was expedited in the present work by the addition of H_2O_2. Figure 1 shows the immediate disappearance of specific fluorescence after the addition of H_2O_2. 'Blank' values obtained in this manner were found to be identical with those obtained by storing the 5HT solution for 64 hr.

The specificity of this method appears to exclude all compounds other than 5-hydroxyindolealkylamines, and it is therefore unlikely that any compound other than 5HT contributes to the final value obtained. It has not been ascertained that the values of 5HT in blood obtained by this method are maximal. However, they are reproducible; replicates of four estimations have been carried out on the same samples of blood and each value was found to vary by less than ±5% from the mean.

Preliminary results show this procedure to give higher recoveries of 5HT bound to tissues than standard methods at present in use.

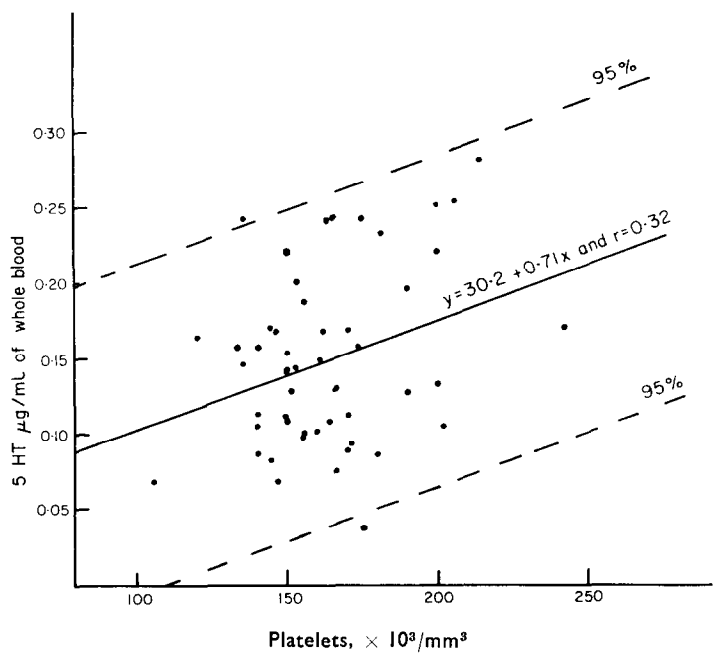

FIG. 2. Relationship between 5HT and blood platelets.

It is interesting to confirm that a correlation exists between 5HT level and blood platelet concentration (Fig. 2). Although 5HT level per unit concentration of platelets has always been assumed to be the most accurate means of expressing the amount of circulating amine, for most clinical purposes the value of 5HT per ml of whole

blood is adequate. If, however, it is necessary to know the 5HT values for 'platelet-rich' and 'platelet-free' plasma, then this method could equally be used for these assays.

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