Rapid method for the determination of 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in small regions of rat brain

G. CURZON AND A. R. GREEN

Department of Chemical Pathology, Institute of Neurology, Queen Square, London W.C.1

A rapid and sensitive method for measuring 5-hydroxytryptamine and 5-hydroxyindoleacetic acid, using *o*-phthalaldehyde and L-cysteine, is presented, enabling both compounds to be measured in small areas of rat brain.

Until recently no methods had been published for the determination of both 5-hydroxytryptamine (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) in small areas of a single rat brain. The methods of Giacalone & Valzelli (1969); Scapagnini, Vandebroek & de Schaepdryver (1969); Welch & Welch (1969); and Jonson & Lewander (1970) all involved determination of 5-HIAA by its native fluorescence at acid pH. Several hundred milligrams of tissue were needed and areas of brain from several animals had to be pooled for analysis.

Maickel & Miller (1966) showed that 3, 5-substituted indoles form highly fluorescent complexes with *o*-phthalaldehyde (OPT) and used this in a very sensitive 5-HT method (Maickel, Cox, Saillant & Miller, 1968). Recently a method for determination of both 5-HT and 5-HIAA using OPT has been described (Miller, Cox, Snodgrass & Maickel, 1970). Korf & Valkenburgh-Sikkema (1969) improved the sensitivity of the OPT method for 5-HIAA in cerebrospinal fluid or urine by adding cysteine to the reaction mixture. We now report a modification, using cysteine, of the method of Maickel *et al.* (1968) for 5-HT by which 5-HT and 5-HIAA may be determined in about 30 mg of brain tissue with good recoveries. As little as 0.01 μ g 5-HT or 5-HIAA may be measured.

Methods. Male Sprague-Dawley rats, 180–220 g (Animal Suppliers, London) were stunned by a sharp blow across the thorax, decapitated and the brains rapidly removed and placed on a glass plate set

in ice at -25° C, and dissected by the procedure of Glowinski & Iversen (1966) except that the hippocampus was not separated from the midbrain area. Dissected material was stored at -25° C until use.

All chemicals were analytical grade where obtainable. *n*-Butanol was acidified by adding 0.85 ml concentrated HCl to 1 l. of *n*-butanol (Chang, 1964). Solid OPT and L-cysteine (British Drug Houses) were stored at -25° C until use. 0.004% w/v OPT in 10 N HCl, 0.1% w/v cysteine in 0.1 N HCl, 1% w/v cysteine in deionized water, and 0.1% w/v OPT in methanol were all prepared immediately before use.

Initial procedures were essentially those of Maickel *et al.* (1968). Thus whole brain or cortex was homogenized in 10 volumes of cold acidified *n*-butanol using an Ultra Turrax homogenizer. All other areas (weighing less than 300 mg) were homogenized in 3 ml of acidified butanol. After centrifugation for 5 min at 3,000 rev/min, 2.5 ml of the supernatant was pipetted into a 25 ml glass stoppered tube and shaken mechanically for 5 min with 5 ml *n*-heptane and 0.4 ml 0.1 N HCl containing 0.1% L-cysteine. The phases were separated by centrifugation as before and 5 ml of the organic phase retained for the 5-HIAA determination.

To determine 5-HT, 0.1 ml samples of the aqueous phase were pipetted into 12 $\times$ 125 mm test tubes and 0.6 ml of 0.004% OPT in 10 N HCl added. After mixing with a Rota mixer and heating in a boiling water bath for 15 min the tubes were cooled in water and fluorescence measured in micro-cuvettes using a Farrand spectrophotofluorometer. Activation and fluorescent wavelengths were 360 nm and 470 nm (both uncorrected) respectively. Standards were prepared as 60 μ g/ml solutions in deionized water (stored at -25° C), diluted 1:100 for use with 0.1 N HCl containing 0.1% cysteine and 0.1 ml reacted with 0.6 ml of the 0.004% OPT in HCl solution. Blanks were prepared by reacting 0.6 ml of the OPT solution with 0.1 ml HCl + cysteine solution only. This reagent blank gave the same reading as tissue blanks prepared by reacting 0.1 ml of the aqueous phase with 0.6 ml 10 N HCl.

To determine 5-HIAA, the 5 ml of organic phase remaining after the extraction of 5-HT was pipetted into a 25 ml

glass stoppered tube containing 0.6 ml 0.5 M phosphate buffer (pH 7.0) and shaken mechanically for 10 minutes. After centrifuging for 3 min at 3,000 rev/min, two 0.2 ml portions of the aqueous phase were pipetted into two test tubes, *A* and *B*. To *A* was added 0.02 ml 1% cysteine solution and to *B* 0.02 ml of 0.02% sodium periodate solution. Then 0.4 ml concentrated HCl was added to both *A* and *B*. After this 0.02 ml of OPT solution (0.1% in methanol) and 0.02 ml periodate solution was added to tube *A*. After 30 min, 0.02 ml of the cysteine and OPT solutions were added to tube *B*. The tubes were then placed in a boiling water bath for 10 min, cooled in water and read at activation: 360 nm fluorescence: 470 nm (both uncorrected). The blank reading (tube *B*) was subtracted from the test reading (tube *A*). Standards were prepared as 15 µg/ml solutions (stored at -25° C) and were diluted 1:100 in the pH 7.0 phosphate buffer for use, 0.2 ml being added to tubes *A* and *B*.

Results and Discussion. Activation and fluorescence spectra derived from both 5-HT and 5-HIAA were essentially as described by Maickel *et al.* (1968) for the 5-HT-OPT complex. The relation between fluorescence and the amount of 5-HT and 5-HIAA present was linear over the range 0.010–2.5 µg with duplicates which agreed within $\pm 1\%$ of the mean for both 5-HT and 5-HIAA. 0.025 µg 5-HT and 0.010 µg 5-HIAA gave a reading which was twice that of the blank fluorescence.

In the presence of cysteine, the fluorescence of the 5-HT reaction mixture was increased about four-fold, as has been shown for 5-HIAA (Korf & Valkenburgh-Sikkema, 1969). Recovery of both 5-HT and 5-HIAA added to the butanol was 95–105% (compared with 55–65% for 5-HIAA in the method of Miller *et al.*, 1970).

With the concentrations found in normal rat brain, interference by 5-HT in the

5-HIAA determination was not found. However 1 µg 5-HT caused an error of about 8% in the determination of 0.25 µg 5-HIAA. This interference could be eliminated by an acid wash which involved shaking 6 ml. of the butanol-heptane phase with 0.3 ml 0.01 N HCl in a 25 ml stoppered tube for 3 min, centrifuging at 3,000 rev/min for 3 min and extracting 5 ml of the organic phase with pH 7.0 phosphate buffer as above. Using the 5-HIAA result the slight interference of 5-HIAA with the 5-HT determination (Maickel *et al.*, 1968) could be corrected for, if required.

Fluorescence due to non-specific contribution from the tissue in the 5-HIAA method was determined using periodate as described by Korf & Valkenburgh-Sikkema (1970). In our study, however, this blank was the same for all areas of brain studied as that found with standard solutions.

The method was rapid and both 5-HT and 5-HIAA could be determined in eighteen rat brains or areas of brains, after removal and separation, in a period of 2.5 hours.

Table 1 shows 5-HT and 5-HIAA contents of different regions of rat brain. Different dissection procedures and source of animals make complete comparison impossible. However, the 5-HT results are in general agreement with, although somewhat higher than those of, Maickel *et al.* (1968) and the 5-HIAA results a little lower than those of Miller *et al.* (1970). The values for whole brain calculated from the average concentrations and weights of each area agree well with our previous determinations for 5-HIAA (Curzon & Green, 1968; Green & Curzon, 1968), when the Giacalone & Valzelli (1966) method was used, though the 5-HT levels are about 20% higher than in our previous work when the Snyder, Axelrod & Zweig (1965) 5-HT assay was used. All rats used in our work were from the same source and were killed at the same time of day

TABLE 1. *5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in regions of rat brain*

	Cortex	Midbrain	Pons medulla	Cerebellum	Striatum	Hypothal- amus	Whole brain
5-HT	0.52±0.03	1.03±0.10	1.24±0.10	0.39±0.08	1.36±0.23	2.64±0.26	0.83
5-HIAA	0.198± 0.026	0.51±0.05	0.54±0.04	0.175± 0.014	0.69±0.08	1.07±0.09	0.35

Each value (in µg/g tissue wet weight) is the mean $\pm$ S.D. derived from seven to eight animals.

to minimize variations due to diurnal changes in brain 5-HT (Scheving, Harrison, Gordon & Pauly, 1968).

Noradrenaline determinations were not made but could presumably have been carried out on the 0.1 N HCl extract by the method of Miller *et al.* (1970).

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