Short communications

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, Vandenbroek & 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 μg 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 12×125 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 μg/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 ±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	Hypothalamus	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±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).

We thank the Mental Health Research Fund for financial support.

REFERENCES

CHANG, C. C. (1964). A sensitive method for spectrophotofluorimetric assay of catecholamines. *Int. J. Neuropharmac.*, **3**, 643–649.

CURZON, G. & GREEN, A. R. (1968). Effect of hydrocortisone on rat brain 5-hydroxytryptamine. *Life Sci., Oxford*, **7**, 657–663.

GIACALONE, E. & VALZELLI, L. (1966). A method for the determination of 5-hydroxyindoyl-3-acetic acid in brain. *J. Neurochem.*, **13**, 1265–1266.

GIACALONE, E. & VALZELLI, L. (1969). A spectrofluorometric method for the simultaneous determination of 2-(5-hydroxyindol-3-yl) ethylamine (serotonin) and 5-hydroxyindol-3-yl-acetic acid in the brain. *Pharmacology*, **2**, 171–175.

GLOWINSKI, J. & IVERSEN, L. L. (1966). Regional studies of catecholamines in the rat brain. I. The disposition of [^{3}H]-norepinephrine, [^{3}H]-dopamine and [^{3}H]-dopa in various regions of the brain. *J. Neurochem.*, **13**, 655–669.

GREEN, A. R. & CURZON, G. (1968). Decrease of 5-hydroxytryptamine in the brain provoked by hydrocortisone and its prevention by Allopurinol. *Nature, Lond.*, **220**, 1095–1097.

JONSON, J. & LEWANDER, T. (1970). A method for the simultaneous determination of 5-hydroxy-3-indole acetic acid (5HIAA) and 5-hydroxytryptamine (5HT) in brain tissues and cerebrospinal fluid. *Acta physiol. scand.*, **78**, 43–51.

KORF, J. & VALKENBURGH-SIKKEMA, T. (1969). Florimetric determination of 5-hydroxyindoleacetic acid in human urine and cerebrospinal fluid. *Clin. chim. Acta*, **26**, 301–306.

MAICKEL, R. P. & MILLER, F. P. (1966). Fluorescent products formed by reaction of indole derivatives and o-phthalaldehyde. *Analyt. Chem.*, **38**, 1937–1938.

MAICKEL, R. P., COX, R. H., SAILLANT, J. & MILLER, F. P. (1968). A method for the determination of serotonin and norepinephrine in discrete areas of rat brain. *Int. J. Neuropharmac.*, **7**, 275–281.

MILLER, F. P., COX, R. H., SNODGRASS, W. R. & MAICKEL, R. P. (1970). Comparative effects of p-chlorophenylalanine, p-chloroamphetamine and p-chloro-N-methylamphetamine on rat brain norepinephrine, serotonin and 5-hydroxyindole-3-acetic acid. *Biochem. Pharmac.*, **19**, 435–442.

SCAPAGNINI, V., VANDENBROEK, R. & DE SCHAEPDRYVER, A. (1969). Simultaneous estimation of 5-hydroxytryptamine and 5-hydroxyindol-3-acetic acid in rat brain. *Biochem. Pharmac.*, **18**, 938–940.

SCHEVING, L. E., HARRISON, W. H., GORDON, P. & PAULY, J. E. (1968). Daily fluctuation (circadian and ultradian) in biogenic amines of the rat brain. *Am. J. Physiol.*, **214**, 166–173.

SNYDER, S. H., AXELROD, J. & ZWEIG, M. (1965). A sensitive and specific fluorescence assay for tissue serotonin. *Biochem. Pharmac.*, **14**, 831–835.

WELCH, A. S. & WELCH, B. L. (1969). Solvent extraction method for simultaneous determination of norepinephrine, dopamine, serotonin and 5-hydroxyindoleacetic acid in a single mouse brain. *Analyt. Biochem.*, **30**, 161–179.

(*Received May* 1, 1970)