Pharmacology Biochemistry & Behavior, Vol. 8, pp. 515–519. Printed in the U.S.A.

Method for the Rapid Determination of Norepinephrine, Dopamine, and Serotonin in the Same Brain Region

DAVID M. JACOBOWITZ[1] AND J. STEVEN RICHARDSON[2,3]

Laboratory of Clinical Science, National Institute of Mental Health, Building 10, Room 2D-46 Bethesda, MD 20014

(Received 18 November 1977)

JACOBOWITZ, D. M. AND J. S. RICHARDSON. *Method for the rapid determination of norepinephrine, dopamine, and serotonin in the same brain region.* PHARMAC. BIOCHEM. BEHAV. 8(5) 515–519, 1978. – A method is presented for the fluorometric analysis of norepinephrine, dopamine and serotonin. This procedure is a combination of an unpublished catecholamine assay developed by Hogans and of the o-phthaldialdehyde serotonin reaction reported by Maickel and Miller [9]. This procedure should greatly facilitate the correlation of neurotransmitter levels in brain regions with changes in behavior produced by experimental manipulations.

Brain Fluorometric assay Dopamine Norepinephrine Serotonin

INTEREST in neurotransmitter activity in the body has made it necessary to analyze several neurotransmitter chemicals in the same tissue sample. We present here a simple and rapid procedure for determining the level of dopamine (DA), norepinephrine (NE) and serotonin (5-HT) in small tissue samples dissected from brain. This method is based on an unpublished catecholamine assay developed by Dr. A. Hogans (unpublished, quoted in Porter *et al.* [12] and Jacobowitz *et al.* [3]) and on the reaction of ortho-phthaldialdehyde and 5-HT reported by Maickel and Miller [9].

METHOD

Dissection

Male Sprague Dawley rats weighing 200–300 g were decapitated, the brains were removed and the hypothalamus, cerebellum, pons-medulla, midbrain, hippocampus, caudate-putamen, and cortex were frozen in liquid nitrogen. Store the brain parts in polyethylene vials at dry ice or liquid nitrogen temperatures until time of assay.

Materials

Reagent grade n-butanol and heptane have usually been found to be satisfactory without further purification.

The 0.1 M phosphate buffer (pH = 6.5) is prepared fresh every 2 or 3 days by adding 32 mls of Stock A (7.1 g sodium phosphate dibasic [Na_2HPO_4] up to 500 mls with distilled water) and 68 mls of Stock B (6.9 g sodium phosphate monobasic [NaH_2PO_4] up to 500 mls with distilled water). Refrigerated, Stock A and Stock B may be stored for up to 6 months.

Versene is prepared by dissolving 4 g disodium ethylene-diaminetetra-acetic acid (EDTA) in 95 mls of distilled water and adjusting the pH to between 6.0 and 6.5 with 10 N sodium hydroxide before making the volume up to 100 mls. Veresene is stored in a refrigerator.

The iodine solution is prepared from 4.8 g potassium iodide plus 0.25 g iodine up to 100 mls with distilled water. The iodine solution should be protected from the light and stored in a refrigerator.

The alkaline sulfite is made fresh daily by dissolving 2.5 g sodium sulfite (Na_2SO_3) in 100 mls 4 N sodium hydroxide.

The OPT solution is prepared fresh daily by dissolving 50 mg of ortho-phthaldialdehyde in 100 mls of absolute methanol. If the supply of OPT crystals are not white to pale yellow thin needles, purify as outlined by Komesu and Thompson [5]. Dissolve several grams of OPT in heptane, heat in a hot (not boiling) water bath and stir. Add 1 g activated charcoal for each 10 mls of solution and stir. Filter using a warm glass funnel into a warm beaker. Place in the dark until maximum recrystalization has occurred (10 min to 2 hr), decant the heptane solution and dry the

[1] Please send reprint requests to Dr. D. M. Jacobowitz.

[2] Fellow, Foundations' Fund for Research in Psychiatry Grant 72-536.

[3] Present Address: Departments of Pharmacology and Psychiatry, College of Medicine, University of Saskatchewan, Saskatoon, Canada, S7N OWO.

TABLE 1

VOLUME OF 0.01 N HCl FOR TISSUES IN A GIVEN WEIGHT RANGE

Weight in g between	HCl in ml	Weight in g between	HCl in ml
0.0000 & 0.0071	0.75	0.2500 & 0.2643	0.57
0.0071 & 0.0214	0.74	0.2643 & 0.2786	0.56
0.0214 & 0.0357	0.73	0.2786 & 0.2929	0.55
0.0357 & 0.0500	0.72	0.2929 & 0.3071	0.54
0.0500 & 0.0643	0.71	0.3071 & 0.3214	0.53
0.0643 & 0.0786	0.70	0.3214 & 0.3357	0.52
0.0786 & 0.0929	0.69	0.3357 & 0.3500	0.51
0.0929 & 0.1071	0.68	0.3500 & 0.3643	0.50
0.1071 & 0.1214	0.67	0.3643 & 0.3786	0.49
0.1214 & 0.1357	0.66	0.3786 & 0.3929	0.48
0.1357 & 0.1500	0.65	0.3929 & 0.4071	0.47
0.1500 & 0.1643	0.64	0.4071 & 0.4214	0.46
0.1643 & 0.1786	0.63	0.4214 & 0.4357	0.45
0.1786 & 0.1929	0.62	0.4357 & 0.4500	0.44
0.1929 & 0.2071	0.61	0.4500 & 0.4643	0.43
0.2071 & 0.2214	0.60	0.4643 & 0.4786	0.42
0.2214 & 0.2357	0.59	0.4786 & 0.4929	0.41
0.2357 & 0.2500	0.58	0.4929 & 0.5000*	0.40

*Weights between 0.5 and 1 g, divide weight by 2, find HCl for 1/2 original weight, put twice this amount of HCl in 10 ml butanol (e.g. tissue weighs 0.7100 ÷ 2 = 0.3550, HCl for 0.3550 = 0.50 ml × 2 = 1 ml HCl in 10 ml butanol). For weights between 1 & 1.5 g, find HCl for 1/3 original weight, put 3 times this amount of HCl in 15 ml butanol.

crystals in a dessicator. When dry, store the pale yellow to white needle-like crystals in an amber or foil-covered bottle at −20° C.

Assay

NE, DA, and 5-HT were assayed as follows. Using a 5 or 10 ml Repipet (Lab Industries, Calif.) pipette 5 ml of butanol into a homogenizing tube and place in ice water. Rapidly weigh each tissue sample insuring that the tissue remains frozen. Add the frozen tissue sample to the butanol and an appropriate amount of 0.01 N HCl (see Table 1), so that the tissue water plus 0.01 N HCl equals 0.75 ml (assuming tissue weight is 70% water) in 5 ml of butanol. Tissue that is too small to weigh without thawing is weighed fresh after dissection from the brain at the time of sacrifice. If tissue sample weighs more than 0.5 g, see footnote Table 1. Homogenize the tissue samples in glass homogenizing tubes with a motor driven teflon pestle (e.g. Arthur Thomas Tissue Grinder size A; or Kontes, Potter-Elvehjem size 22). Holding the homogenizing tube in ice water, homogenize until the tissue is uniformly distributed throughout the butanol (1 to 2 min depending on weight of tissue). Pour the homogenate into a 12 ml conical centrifuge tube and centrifuge (IEC Model K, 1500 rpm) for 10 min to sediment the tissue debris leaving a clear supernatant.

Aliquot 2 ml of butanol supernatant into a 12 ml conical centrifuge tube containing 1.5 ml (use 5 ml Repipet) of 0.1 M phosphate buffer in ice water. For analysis of 5-HT, aliquot an additional 2 ml of butanol supernatant into a 15 ml glass stoppered centrifuge tube containing 5 ml (5 ml Repipet) heptane and 0.5 ml (1 ml Repipet) of 0.1 N HCl in ice water. Place the ground glass stopper firmly in the tube. Vortex (Vortex Geni) both mixtures for 20 sec. NE and DA are extracted into the phosphate buffer and 5-HT is extracted into the 0.1 N HCl. Centrifuge (3000 rpm) to separate the organic and aqueous layers and, using a vacuum with a liquid trap, aspirate the top organic layer including any tissue disc, leaving as much of the aqueous layer as possible. Aliquot 1 ml (1 ml Biopette, Schwarz-Mann) of the phosphate extract into a 16 × 150 mm test tube. Aliquot 0.3 ml (1 ml Biopette) of the 0.1 N HCl extract into a 16 × 150 mm test tube. These aliquots may be frozen and the assay continued the following day.

Oxidize the NE and DA in the phosphate buffer extract into fluorophores by adding 0.25 ml (1 ml Repipet) Versene and vortex briefly. Then, at *exactly* 2 min intervals, add: (1) 0.20 ml (1 ml Repipet) iodine, vortex briefly, (2) 0.25 ml (1 ml Repipet) alkaline sodium sulfite, vortex briefly and (3) 0.3 ml (1 ml Repipet) 5 N acetic acid, vortex briefly. Place test tubes in boiling water for 5 min, then in ice water for 1 min. Read NE fluorescence immediately in an Aminco-Bowmen spectrophotofluorometer (American Instrument Co., Md) excitation 385/emission 485. Read DA fluorescence at least 20 min later at 320/385. Convert fluorescence reading into μg NE or DA per g of tissue as described below.

The highly fluorescent condensation product of orthophthaldialdehyde and the 5-HT in the 0.1 N HCl extract is prepared by adding 0.2 ml (1 ml Repipet) of OPT solution followed immediately by 1.5 ml (5 ml Repipet) of concentrated (10 N) HCl. Vortex briefly and place test tubes in boiling water for 10 min, then cool to room temperature in tap water. Read 5-HT fluorescence at 360/470 and convert fluorescence reading into μg 5-HT per g tissue as described below.

Standards were prepared and calculations were performed as follows. Mix separate standard stock solutions of NE, DA and 5-HT as 400 μg of base per ml of 0.01 N HCl. These stock solutions are good for about 6 months in the refrigerator. Keep all subsequent solutions in ice water. Take 1 ml (1 ml volumetric pipet) of NE stock (400 μg NE) and 1 ml of DA stock (400 μg DA), place in 10 ml volumetric flask and add 0.01 N HCl to 10 ml (40 μg/ml NE and 40 μg/ml DA) and mix. Transfer 1 ml of this solution (1 ml volumetric pipet) into a 25 ml volumetric flask, add 0.01 N HCl to volume (1.6 μg/ml). Add 2 ml of this solution to a test tube containing 2 ml of 0.01 N HCl and vortex. This is Standard A Stock (0.8 μg/ml). Add 1 ml of Standard A Stock to 3 ml 0.01 N HCl in a test tube, vortex. This is Standard B Stock (0.2 μg/ml). Take 1 ml of Standard B Stock and add to 3 ml of 0.01 N HCl in a test tube and vortex. This is Standard C Stock (0.05 μg/ml). Add 0.5 ml of A Stock and 0.25 ml of 0.01 N HCl to 5 ml of butanol in a 12 ml conical centrifuge tube and vortex for 20 sec; this is A Standard (0.4 μg). Add 0.5 ml of B Stock and 0.25 ml of 0.01 N HCl to 5 ml butanol, vortex. This is B Standard (0.1 μg). Add 0.5 ml of C Stock and 0.25 ml 0.01 N HCl to 5 ml butanol, vortex. This is C Standard (0.025 μg).

Take two untreated rats of a size, sex and strain similar

TABLE 2

CALCULATIONS AND REPRESENTATIVE STANDARD VALUES

Tube	(NE Reading)	–	(NE Blank)	÷	(μg NE Added)	=	NE Slope
A	390		18		0.4		930
B	108		11.4		0.1		966
C	33		8.4		0.025		984
						$\bar{X}$ NE Slope	960

Tube	(DA Reading)	–	(DA Blank)	÷	(μg DA Added)	=	DA Slope
A	560		40		0.4		1300
B	165		35		0.1		1300
C	65		34		0.025		1240
						$\bar{X}$ DA Slope	1280

Tube	(5HT Reading)	–	($\bar{X}$ Reagent Blank)	÷	(μg 5HT Added)	=	5HT Slope
X	350		16.4		0.4		834
Y	96		16.4		0.1		796
Z	35		16.4		0.025		744
R_1	16.5					$\bar{X}$ 5HT Slope	791
R_2	16.2						

TISSUE CALCULATIONS

NER – NEB = NE Corr ÷ $\bar{X}$ NE Slope ÷ Wt in g = NE μg/g

Tissue	Wt.	NER	NEB	NE Corr.	$\bar{X}$ NE Slope	NE μg/g
Hypothalamus	0.0457	96	10.5	85.5	960	1.95
Hippocampus	0.1026	50	9.2	40.8	960	0.41
Caudate Putamen	0.1276	48	9.8	38.2	960	0.31

to those used in the experiment. Decapitate and dissect the various brain regions. Freeze, weigh and homogenize brain parts and centrifuge homogenates as described above. Place a 4 ml aliquot of the clear butanol supernatant from the A, B and C Standards and from these tissue samples into separate centrifuge tubes containing 3 ml of the phosphate buffer. Vortex 20 sec, centrifuge and aspirate the top organic phase including any tissue disc leaving at least 2 ml of the bottom phosphate extract. Transfer duplicate 1 ml aliquots (1 ml Biopette) into two test tubes labeled to identify initial and duplicate aliquot. One aliquot is oxidized into a fluorophore as above; the second aliquot is processed to provide a blank reading by adding, at exactly 2 min intervals, first the alkaline sulfite, then the iodine and finally the acetic acid. By reversing the order in which the iodine and the alkaline sulfite are added, the oxidation of NE and DA into fluorophores is prevented. Boil, cool and read as above.

Subtract the blank fluorescence reading of the standards from their corresponding oxidized fluorescence reading. Average the blank readings for a particular brain area and subtract this mean blank value from each oxidized reading of similar areas from the brains of the experimental and control rats. Divide the corrected (i.e. blank subtracted) fluorescence reading of each Standard by the amount of added NE or DA (i.e. corrected A ÷ 0.4 = x_1, corrected B ÷ 0.1 = x_2, corrected C ÷ 0.025 = x_3) and calculate the average slope [($x_1 + x_2 + x_3$) ÷ 3 = average slope]. Divide each corrected tissue fluorescence reading by the average slope to determine μg NE or μg DA per sample and divide this by the weight in g of the particular tissue sample to determine the amount of NE or DA per g of tissue. See Table 2 for an example of these calculations.

Prepare 5-HT standards in a similar manner (1 ml of 400 μg/ml stock up to 10 ml, 1 ml of this up to 25 ml, 2 ml of this plus 2 ml 0.01 N HCl is Standard X Stock; 1 ml X Stock plus 3 ml of 0.01 N HCl is Standard Y Stock; 1 ml Y Stock plus 3 ml 0.01 N HCl is Standard Z Stock; 0.5 ml of each X, Y and Z Stocks + 0.25 ml 0.01 N HCl each into separate centrifuge tubes containing 5 ml of butanol). Make two reagent blanks by adding 0.75 ml of 0.01 N HCl to 5 ml of butanol. Process these 5-HT Standards [X Standard (0.4 μg), Y Standard (0.1 μg) and Z Standard (0.025 μg)] and reagent blanks (R_1 and R_2) as butanol homogenates as in the assay. React with OPT and read as above. Average the reagent blank fluorescence readings and subtract this mean blank value from the fluorescence readings of all of the Standards and all of the tissues. Divide the corrected

TABLE 3

REPRESENTATIVE VALUES FOR VARIOUS BRAIN AREAS (DATA REPRESENT THE MEAN ± SEM FOR 5 TO 7 RATS)

Tissue	Weight in g	NE μg/g	DA μg/g	5-HT μg/g
Hypothalamus	0.0466 ± 0.003	1.96 ± 0.056	0.30 ± 0.018	2.03 ± 0.125
Hippocampus	0.1249 ± 0.009	0.27 ± 0.018	–	0.57 ± 0.056
Caudate-Putamen	0.1073 ± 0.004	0.33 ± 0.023	4.90 ± 0.162	1.05 ± 0.069
Midbrain	0.2365 ± 0.003	0.43 ± 0.012	0.13 ± 0.013	1.10 ± 0.033
Cortex	0.7695 ± 0.019	0.14 ± 0.005	0.21 ± 0.011	0.49 ± 0.027
Cerebellum	0.2634 ± 0.006	0.20 ± 0.012	–	0.33 ± 0.015
Pons-Medulla	0.2062 ± 0.002	0.43 ± 0.029	–	1.11 ± 0.128

Standard readings by the amount of 5-HT in each (corrected X reading ÷ 0.4, Y ÷ 0.1, Z ÷ 0.025) and calculate the average slope. Divide each corrected tissue reading by this average slope to determine total 5-HT in the sample and then by the weight of the sample in grams to give 5-HT per gram tissue. See Table 2 for an example of these calculations. Prepare one set of three Standards for each 40 tissue samples assayed.

If only DA and NE are to be assayed, put a 4 ml aliquot of the clear butanol supernatant in 3 ml of phosphate buffer and proceed as described for the preparation of the A, B and C standards. Thus each tissue sample will provide a duplicate phosphate extract aliquot that will become its own tissue blank value.

If the experimental manipulation might be expected to result in significant brain levels of 5-hydroxytryptophan (5HTP), then it is necessary to remove the 5HTP before the heptane and 0.1 N HCl step by washing the clear butanol supernatant aliquot twice with 2 volumes of borate buffer prepared as follows. Wash 100 ml butanol in equal volumes of (1) 0.1 N NaOH, separate; (2) 0.1 N HCl, separate; (3) distilled water, separate; and (4) distilled water a second time, separate. Add 94.2 g boric acid to 3 liters distilled water in a large beaker. Mix. Add 165 ml of 10 N NaOH. Saturate with the washed butanol (about 50 ml) and add excess NaCl (about 2 kg). Let NaCl cyrstals settle, aspirate excess butanol, decant buffer from excess NaCl and store in a large bottle.

RESULTS AND DISCUSSION

The original catecholamine assay as developed by Hogans has been used extensively for several years processing many thousands of tissue samples [2, 3, 4, 6, 7, 8, 11, 12]. Representative values for brain parts from non-treated rats dissected and assayed for DA, NE and 5-HT according to the procedure outlined above are presented in Table 3. While it is possible to process with ease about 50 samples of frozen tissues per day, it has been found that, by processing the samples through to the phosphate buffer for catecholamines or the 0.1 N HCl extract for 5-HT and freezing these extracts (−20° C) for fluorophore formation later, it is possible to increase the average daily production rate to about 100 tissue samples per day.

Percent recovery for this assay depends on tissue size and compares favorably with the percent recovery reported for other assays. For small samples (e.g. hypothalamus, about 50 mg) recovery is 90 to 100% while as tissues approach 500 mg, recovery approaches 50%.

This is a very useful assay procedure especially in any experiment that generates many samples. This would include experiments such as dose-response or time course analyses of drug action [14], or neurotransmitter correlates of behavior [10,13]. This procedure has been extended to the analysis of 5-HT in platelets [1].

REFERENCES

1. Costa, J. L., J. S. Richardson and D. L. Murphy. Comparative evaluation of a platelet serotonin assay utilizing ortho-phthaldialdehyde for fluorophore production. *Throm. Diath. Haemorrhagia* **33:** 130–131, 1975.
2. Goldman, H. and D. M. Jacobowitz. Correlation of norepinephrine content with observations of adrenergic nerves after a single dose of 6-hydroxydopamine in the rat. *J. Pharmac. exp. Ther.* **176:** 119–133, 1971.
3. Jacobowitz, D. M., T. Cooper and H. B. Barner. Histochemical and chemical studies of the localization of adrenergic and cholinergic nerves in normal and denervated cat hearts. *Circ. Res.* **20:** 289–298, 1967.
4. Jacobowitz, D. M. and R. M. Kostrzewa. Selective action of 6-hydroxydopa on noradrenergic terminals: Mapping of preterminal axons of the brain. *Life Sci.* **10:** 1329–1342, 1971.

5. Komesu, N. N. and J. H. Thompson. Optimal concentration of orthophthaldialdehyde for the fluorometric measurement of serotonin. *Eur. J. Pharmac.* **16**: 248–250, 1971.
6. Kostrzewa, R. M. and J. W. Harper. Effect of 6-hydroxydopa on catecholamine-containing neurons in brains of newborn rats. *Brain Res.* **69**: 174–181, 1974.
7. Kostrzewa, R. M. and D. M. Jacobowitz. The effect of 6-hydroxydopa on peripheral adrenergic neurons. *J. Pharmac. exp. Ther.* **183**: 284–297, 1972.
8. Kostrzewa, R. M. and D. M. Jacobowitz. Acute effects of 6-hydroxydopa on central monoaminergic neurons. *Eur. J. Pharmac.* **21**: 70–80, 1973.
9. Maickel, R. P. and F. P. Miller. Fluorescent products formed by reaction of indole derivatives and o-phthalaldehyde. *Anal. Chem.* **38**: 1937–1938, 1966.
10. Marshall, J. F., J. S. Richardson and P. Teitelbaum. Nigrostriatal bundle damage and the lateral hypothalamic syndrome. *J. comp. physiol. Psychol.* **87**: 808–830, 1974.
11. Nichols, C. W., D. M. Jacobowitz and M. Hottenstein. The influence of light and cold on the catecholamine content of the retina and choroid. *Invest. Opthalmol.* **6**: 642–646, 1967.
12. Porter, C. C., J. A. Totaro and A. Burcin. The relationship between radioactivity and norepinephrine concentrations in the brains and hearts of mice following administration of labelled methyldopa or 6-hydroxydopamine. *J. Pharmac. exp. Ther.* **150**: 17–22, 1965.
13. Richardson, J. S., N. Cowan, R. Hartman and D. M. Jacobowitz. On the behavioral and neurochemical actions of 6-hydroxydopa and 5,6-dihydroxytryptamine in rats. *Res. Communs chem. pathol. Pharmac.* **8**: 29–44, 1974.
14. Richardson, J. S. and D. M. Jacobowitz. Depletion of brain norepinephrine by intraventricular injection of 6-hydroxydopa: A biochemical, histochemical and behavioral study in rats. *Brain Res.* **58**: 117–133, 1973.