PHARMACOLOGICAL REVIEWS
Vol. 18, No. 1, Part I

Printed in U.S.A.

C. NEWER DEVELOPMENTS IN CATECHOLAMINE ASSAY

JAN HÄGGENDAL

Department of Pharmacology, University of Göteborg

At the Symposium in 1958 methods for the assay of catecholamines were discussed and some new methods were also presented. Since then these methods have been further developed. Today almost every laboratory working in the catecholamine field has its own modification of some method according to the main principles for catecholamine assay. This development appears to be natural since the catecholamines can be assayed in different ways with good results. In many laboratories the researchers appear to have modified the method they once started with; they have become experts in this method and in the hands of these trained people the particular method can give excellent results. It is impossible here to give more than a brief summary of some of the most important modifications and improvements presented during the last years (for reviews see 5, 11, 13, 32).

The main steps in the chemical assay of catecholamines are purification and concentration of the amines and thereafter the final estimation (most often conversion of the amines to fluorescent compounds followed by fluorometry). Before assay, the biological material must be prepared and if necessary an extract must be made. The proteins are precipitated, *e.g.*, with acid alcohol, trichloroacetic acid or perchloric acid.

The rate of post mortem loss of catecholamines has been studied by Bertler and Rosengren (4), and others. After 1 hr 84% norepinephrine (NE) and 74% dopamine were found in rabbit brains compared to those immediately extracted. The decrease of dopamine in the caudate nucleus and of NE in hypothalamus in human brain was slow (15). It appears, however, that the decrease is faster in brain areas where the catecholamine amounts are low (24). Losses of NE in cat muscle have been reported already within 10 min after death (25). Significantly higher values of NE in cat heart and brain were reported following freezing in liquid nitrogen than after acid homogenization (6). The values reported after homogenization of brain tissue in acid are, however, low compared with values from other laboratories. Care must be taken to keep the extract cold (about 0°C) during the homogenization.

The stability of NE in a perchloric acid extract is lower at a pH about 0.5 than at pH 3.8 (16). This is also true for 5-hydroxytryptamine (16) and probably also for dopamine. Falsely high values of the free catecholamines may occur if strongly acidified urine is kept at room temperature, owing to hydrolysis of the conjugated catecholamines (14).

The purification and concentration procedure can be performed in different ways. Adsorption on alumina either by mixing or by passage through a column is frequently used. In addition to NE and E also other catechols, such as dopamine, dopa, dihydroxyphenylacetic acid and dihydroxymandelic acid present in the extracts, will be adsorbed and recovered. This method has great advantages

since it is rather simple and can be used also when the ionic strength varies, as in urine. When small amounts of catecholamines are assayed, a fine precipitate of alumina salts may interfere. A gelatinous calcium-magnesium-phosphate precipitate which can quench the fluorescence markedly is eliminated by the addition of EDTA (10). A simple and much used technique is the solvent extraction method presented by Shore at the Symposium in 1958 (27). The tissue can be homogenized directly in butanol.

Several chromatographic procedures are available (for review see 11 and others). Paper chromatography has been used for separation but also for identification. With thin layer chromatography using polyamide for the simultaneous separation of catecholamines and their derivatives a better sensitivity has been reported than with paper chromatography. The sensitivity has been reported to be below 3 ng for E (26). These last procedures generally require purification and concentration of the tissue extract prior to the chromatography.

Columns of weak cation exchange resins have been employed. They are used for separation of normetanephrine and metanephrine after suitable pretreatment. They are not suitable for extracts with high ionic strength. In this respect the strong cation exchange resins are superior. With such columns NE and E were separated from dopamine (2). By lengthening the column a complete separation can be obtained between NE, E, and dopamine, and their 3-O-methylated derivatives (17). Such separation is useful for identification purposes, and even the nonspecific native fluorescence can be used for quantitative analysis. A total separation is, however, somewhat time-consuming and may give some destruction of the catecholamines. The losses may be controlled by means of antioxidants and metal complexing agents. In the assay of small amounts of catecholamines it must be observed that fluorescent material is given off from the strong cation exchange resin. This is of importance for the choice of blanks and may also explain why in some cases de-ionized water has caused difficulties at the assay.

The shape of the columns can be altered to obtain the catecholamines in small eluate volumes. It is not always necessary to separate NE and E. Dopamine can easily be obtained in a separate eluate on a short column (2). Different batches of resin often vary somewhat, *e.g.*, with respect to the particle size. The precise operation of a column prepared from a new batch of resin is therefore not predictable. In our laboratory new columns are always tested by adding fairly large amounts of catecholamines, *e.g.*, 5 to 10 μg each of NE and dopamine. Fractional elution is then performed and the native fluorescence of each fraction is measured. On the basis of these data the elution volumes suitable for routine work are obtained. The elution is performed with strong acids, *e.g.*, hydrochloric acid, but may also be done with weakly alkaline solutions. The columns are rather simple to handle and can be used several times after washing.

Gas chromatography appears at present not to be suitable for routine assay of catecholamines since the analysis of biological material presents many difficulties. The trimethylsilyl ethers of the amines (21) appear to be promising derivatives for estimation.

For the chemical determination of small amounts of catecholamines only fluorimetric methods appear at present to be sensitive enough. Among methods available the trihydroxyindole reaction appears to be more specific than the ethylenediamine condensation reaction (*e.g.*, 13, 30). The latter reaction, discussed at the Symposium in 1958 (31), may prove useful after chromatographic separation. The reaction has recently been used in a sensitive procedure for dopamine estimation (20). Some metabolites of the catecholamines can also be assayed according to the principles of this reaction.

The reaction most frequently used, however, is the trihydroxyindole method. The principal reactions in this method are well known and were discussed at the Symposium in 1958 by Euler (12). Here some problems will be mentioned particularly with respect to the assay of very small amounts of catecholamines. The oxidation of NE and E to chrome derivatives can be performed in different ways, *e.g.*, by iodine or potassium ferricyanide. An advantage of potassium ferricyanide in the concentrations generally used is that dopamine, normetanephrine and metanephrine, if present, will hardly interfere. In general the oxidation is interrupted and the chrome derivatives converted into fluorescent lutines by the addition of a mixture of ascorbic acid and sodium hydroxide; but the ascorbic acid appears to be a weak point in the reaction, and several modifications have been proposed. The blanks obtained are not stable but increase with time. The blanks are also rather high. This is of particular importance when the sample contains small amounts of catecholamines, as in plasma.

Several compounds have been tried for stabilizing the lutines. After addition of ethylene diamine or propylene diamine to the alkali-ascorbic acid mixture the blank readings are stable for several hours (14). It has been reported, however, that ethylene diamine was not satisfactory at low concentrations of catecholamines; instead β-thiopropionic acid was added to the ascorbic acid mixture (23). Substitutes for the ascorbic acid have been tried for the stabilization (19). Some sulphur-containing compounds were found useful; among the best were dimercaptopropanol (BAL) and cysteine hydrochloride. When the oxidation was performed with potassium ferricyanide in the presence of Cu^{++} ions, we obtained lower and more stable blanks with BAL-sodium sulphite-sodium hydroxide mixtures than with any other procedures which we have tried. Furthermore two activating peaks were found for NE and E: the well known peaks at about 400 mμ and other peaks at 330 mμ. With earlier methods the latter peaks were masked by the addition of ascorbic acid. The presence of two activating peaks has proved to be of value for identification, particularly when the intensity of the fluorescence is weak.

In the assay of small amounts of catecholamines, much attention must be paid to the tissue blanks. Not only the magnitude and the stability of the blank are of importance, but also the choice of the procedure when the blank is prepared. Generally the "faded" tissue blank (obtained by adding the antioxidant after instead of together with the alkali) is lower than the "nonoxidized" tissue blank (obtained by adding the oxidant after the antioxidant). This was found both with ascorbic acid and with the BAL-sodium sulphite solution as anti-

oxidants. Part of the difference between the blanks could be explained by the fluorescent material released from the strong cation exchange resin. More of the fluorescent material was destroyed in the "faded" blank than in the "nonoxidized" blank or in the sample. The reading of the faded blank thus tends to be too low. The interference by this material could be reduced by washing the columns intensely. The reading of the "nonoxidized" tissue blank generally tends to be too high, partly because of the above-mentioned impurity and partly because of spontaneous oxidation of catecholamines, which can be detected by recording the blank spectra. For calculation the mean values of the two blanks are used in our laboratory.

The differentiation of NE and E can be performed in several ways: (a) by complete separation by paper chromatography, column separation or other procedures before the oxidation; (b) by oxidation at different pH; (c) by using thioglycolic acid as a differential stabilizing reagent for the specific estimation of NE (22); (d) by making use of the differences in the activation and fluorescence spectra of the lutines. When BAL-sodium sulphite is used instead of ascorbic acid the different ratio between the two activating peaks for NE and E can be utilized. Internal standards of both NE and E should be used, however, since the intensity at 330 mμ appears to be more easily reduced by interfering compounds than the intensity at about 400 mμ. The differentiation between NE and E is difficult especially when one of the compounds is present in large amounts compared to the other. In the assay of small amounts the satellite spectra of the light scatter (28) may disturb particularly the E estimation. We have found it valuable to record the spectra routinely when small amounts of catecholamines are assayed.

The sensitivity of the fluorimetric estimation is now on the nanogram level. It is possible to increase the sensitivity by reducing the volumes in the oxidation procedure and by reading in microcuvettes. But in that case also the purification and concentration procedures must accommodate the reduced volumes.

A sensitive estimation procedure for dopamine based on the principles of the trihydroxyindole method (8) was presented at the Symposium in 1958 (9). After oxidation with iodine the final fluorescence was obtained after ultraviolet radiation. The fluorescence was read at pH near 5.3, where the activation and fluorescence peaks differ from those of NE and E. The purification and concentration procedure was performed with strong cation exchange resin. Adsorption on alumina is sometimes used (*e.g.*, 1). For the oxidation periodate is also used (1), or manganese dioxide (29).

The principles of the trihydroxyindole method can be used not only for the estimation of NE, E, and dopamine, but also for their 3-O-methylated derivatives (3, 7). With a strong cation exchange resin column the purification and separation can be performed in one procedure (18). Such columns are also useful for investigations with labeled catecholamines and their basic metabolites.

REFERENCES

1. Anton, A. H. and Sayre, D. F.: The distribution of dopamine and dopa in various animals and a method for their determination in diverse biological material. J. Pharmacol. 145: 326-336, 1964.

2. BERTLER,Å., CARLSSON, A. AND ROSENGREN, E.: A method for the fluorimetric determination of adrenaline and noradrenaline in tissues. Acta physiol. scand. 44: 273–292, 1958.
3. BERTLER,Å., CARLSSON, A. AND ROSENGREN, E.: Fluorimetric method for differential estimation of the 3-O-methylated derivatives of adrenaline and noradrenaline (metanephrine and normetanephrine). Clin. chim. Acta 4: 456–457, 1959.
4. BERTLER,Å. AND ROSENGREN, E.: Occurrence and distribution of catechol amines in brain. Acta physiol. scand. 47: 350–361, 1959
5. CALLINGHAM, B. A. AND CASS, R.: The determination of catecholamines in biological materials. In: The Clinical Chemistry of Monoamines, ed. by H. Varley and A. H. Gowenlock, pp. 19–30, Elsevier Publ. Co., Amsterdam, 1963.
6. CALLINGHAM, B. A. AND CASS, R.: A modification of the butanol extraction method for the fluorimetric assay of catecholamines in biological materials. J. Pharm., Lond. 15: 699–700, 1963.
7. CARLSSON, A.: Detection and assay of dopamine. Pharmacol. Rev. 11: 300–304, 1959.
8. CARLSSON, A. AND LINDQVIST, M.: A method for the determination of normetanephrine in brain. Acta physiol. scand. 54: 83–86, 1962.
9. CARLSSON, A. AND WALDECK, B.: A fluorimetric method for the determination of dopamine (3-hydroxytyramine). Acta physiol. scand. 44: 293–298, 1958.
10. CROUT, J. R.: Catecol amines in urine. In: Standard Methods of Clinical Chemistry, ed. by D. Seligson, vol. 3, pp. 62–80, Academic Press, New York, 1961.
11. EULER, U. S. VON: Noradrenaline. Charles C Thomas, Springfield, Ill., 1956.
12. EULER, U. S. VON: The development and applications of the trihydroxyindole method for catecholamines. Pharmacol. Rev. 11: 262-268, 1959.
13. EULER, U. S. VON: The catecholamines. Adrenaline; noradrenaline. In: Hormones in Blood, ed. by C. H. Gray, pp. 515–582, Academic Press, Inc., New York, 1961.
14. EULER, U. S. VON AND LISHAJKO, F.: Improved technique for the fluorimetric estimation of catecholamines. Acta physiol. scand. 51: 348–355, 1961.
15. GANROT, P. O., ROSENGREN, E. AND GOTTFRIES, C. G.: Effect of iproniazid on monoamines and monoamine oxidase in human brain. Experientia 18: 260–261, 1962.
16. GUNNE, L.-M.: Catecholamines and 5-hydroxytryptamine in morphine tolerance and withdrawal. Acta physiol. scand. 58: suppl. 204, 1963.
17. HÄGGENDAL, J.: On the use of strong exchange resins for determinations of small amounts of catechol amines. Scand. J. clin. Lab. Invest. 14: 537–544, 1962.
18. HÄGGENDAL, J.: Fluorimetric determination of 3-O-methylated derivatives of adrenaline and noradrenaline in tissues and body fluids. Acta physiol. scand. 56: 258–266, 1962.
19. HÄGGENDAL, J.: An improved method for fluorimetric determination of small amounts of adrenaline and noradrenaline in plasma and tissues. Acta physiol. scand. 59: 242–254, 1963.
20. LAVERTY, R. AND SHARMAN, D. F.: The estimation of small quantities of 3,4-dihydroxyphenylethylamine in tissues. Brit. J. Pharmacol. 24: 538–548, 1965.
21. LINDSTEDT, S.: Gas chromatography of adrenalin, noradrenalin and related amines as their trimethylsilyl ethers. Clin. chim. Acta. 9: 309–310, 1964.
22. MERRILLS, R. J.: A semiautomatic method for determination of catecholamines. Analyt. Biochem. 6: 272–282, 1963.
23. PALMER, J. F.: The use of β-thiopropionic acid for the analysis of mixtures of adrenaline and noradrenaline in plasma by the fluorometric trihydroxyindole method. W. I. Med. J. 13: 38–53, 1964.
24. ROSENGREN, E.: Personal communication, 1965.
25. SEDVALL, G.: Noradrenaline storage in skeletal muscle. Acta physiol. scand. 60: 39–50, 1964.
26. SEGURA-CARDONA, R. AND SOEHRING, K.: Dünnschichtchromatographischer Nachweis kleinster Mengen von Katecholaminen und deren Derivaten. Med. exp. 10: 251–257, 1964.
27. SHORE, P. A. AND OLIN, J. S.: Identification and chemical assay of norepinephrine in brain and other tissues. J. Pharmacol. 122: 295–300, 1958.
28. UDENFRIEND, S.: Fluorescence Assay in Biology and Medicine. Academic Press, Inc., New York, 1962.
29. UUSPÄÄ, V. J.: A new method for the determination of dopamine (3-hydroxytyramine). Ann. Med. exp. Fenn. 41: 194–201, 1963.
30. VALK, A. DE T. JR. AND PRICE, H. L.: The chemical estimation of epinephrine and norepinephrine in human and canine plasma. I. A critique of the ethylenediamine condensation method. J. clin. Invest. 35: 837–841, 1956.
31. WEIL-MALHERBE, H.: The fluorimetric estimation of catechol compounds by the ethylenediamine condensation method. Pharmacol. Rev. 11: 278–288, 1959.
32. VENDSALU, A.: Studies on adrenaline and noradrenaline in human plasma. Acta physiol. scand. 49: suppl. 173, 1960.