SPECIAL ARTICLE

A MODIFIED METHOD OF DETERMINING URINARY CATECHOL AMINES*

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It is well established that the adrenal medulla releases small amounts of epinephrine (E) and norepinephrine (NE) when at rest.[1,2] Catechol amine secretion increases in a wide variety of conditions such as stimulation of sympathetic nerves,[1] hypoglycemia,[2,3] emotional stress,[1] electroshock treatment,[5] and hemorrhagic shock.[6,7] The estimation of E and NE in plasma and urine has become an important procedure in scientific studies and in the diagnosis of pheochromocytoma. With the development of fluorometric procedures the determination of trace amounts of E and NE became possible. Most commonly used fluorometric procedures are the trihydroxyindole method and the ethylenediamine condensation method. The sensitivity of the ethylenediamine condensation method is found to be very high so that small amounts of E and NE can be detected, however it is not specific for E and NE.[8,9] The trihydroxyindole method is less sensitive and more specific than the ethylenediamine method.[8]

While working on urinary catechol amines by the method of von Euler and Floding[10] the investigator noticed that some interfering substances were present in the urine.

The purpose of the present study is to describe a method of eliminating these interfering compounds from the urine extract. The method of von Euler and Floding is modified by washing the acid eluate containing the catechols with two 4 ml. aliquots of n-butanol which had previously been purified by washing with 0.1N sodium hydroxide, 0.1N hydrochloric acid and distilled water. A standard solution of NE was made up in 0.25N sulfuric acid. The acid had been treated previously with aluminum oxide. All specimens were centrifuged immediately before fluorescence was determined.

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Material and Methods

Reagents

1. Aluminum oxide — Woelm, Grade I
2. 0.25N $H_2 So_4$
3. 10 % Sodium bicarbonate solution
4. 1 M acetate buffer, pH 3.1
5. 1 M acetate buffer, pH 6.7
6. 0.5 % Zinc sulfate solution
7. 0.25 % Potassium ferricyanide solution
8. 20 % NaOH
9. 0.5N NaOH
10. 2 % Ascorbic acid — must be prepared fresh for each determination and kept cold until used.
11. Bromphenol blue 1 % solution
12. Bromthymol blue 1 % solution
13. Thymol blue test paper, pH 8.2-9.1.
14. N-butanol — washed with equal volumes of 0.1N HCL 0.1N NaOH and twice with distilled water.
15. Ascorbic acid — NaOH solution — To 4.5 ml. of 20 % NaOH add 0.5 ml. 2 % ascorbic acid. This mixture should be made just before using — must be used in 15 minutes or fresh mixture must be made.

Collection of specimens

Twenty-four hour urine specimens are collected in sufficient acid to keep the pH 1-2. Specimens should be refrigerated during and after collection.

Procedure

Measure 24 hour urine volume. If there is any precipitate present it should be filtered. A 100 ml. aliquot of urine is taken for the determination.

Boil 100 ml. of urine in a beaker with frequent stirring for 15 minutes. The pH of the urine should be checked at intervals during boiling and acid should be added if pH rises above 2.

If only the free form of catechol amines is to be determined; the urine is not boiled.

Cool the urine and add distilled water to restore volume to 100 ml. Place beaker on magnetic stirrer and place teflon covered stirring rod in beaker. Add 2.5 Gm. aluminum oxide and 1 - 2 drops of bromthymol blue. With constant stirring, carefully add 20 percent NaOH until indicator color change is noted. Then add 0.5N NaOH to pH 8.5, checking with thymol blue test paper. Continue to stir for 4 minutes.

Transfer to Buchner funnel on suction bottle. Filter with strong suction. If filtration proceeds slowly the supernatant liquid may be aspirated after the alumina has settled on the filter. Wash the alumina twice with 25 ml. of cold distilled water and remove all water by suction and aspiration as above.

Place Buchner funnel on clean suction bottle. Add 5 ml. of 0.25N H_2SO_4 to the alumina on the filter and swirl gently for 3 minutes. Collect acid by suction into bottle. Repeat with 4 ml. 0.25N H_2SO_4.

Place the 9 ml. of acid eluate in a 15 cc. glass-stoppered centrifuge tube. Add 4 ml. of washed n-butanol, shake, centrifuge and aspirate the butanol layer. Repeat with second 4 ml. of n-butanol.

Transfer the acid solution to a glass-stoppered 10 ml. graduated cylinder and add a small drop of bromphenol blue indicator. Carefully adjust to pH 4 by adding 10 per cent sodium bicarbonate. After initial indicator color change check pH with pH test paper. Bring volume to 10 ml. by adding distilled water. Let solution stand in refrigerator for 1 to 2 hours.

Preparation of Standard

Stock solutions of epinephrine and norepinephrine, 20 micrograms per cc. in 0.1N HCL may be kept indefinitely in dark bottles in the refrigerator. Norepinephrine is used as the standard because it occurs in much higher concentration in urine than epinephrine, and it is more stable than epinephrine at pH 6.7.

One - half cc. norepinephrine standard solution, 10 micrograms norepinephrine, is added to 5 ml. 0.25N H_2SO_4 in a glass-stoppered centrifuge tube. This is washed twice with n-butanol as described above and is then transferred to a 10 ml. graduated cylinder. The pH is adjusted to pH 4 with 10 percent sodium bicarbonate in the same manner as described for urine specimens and the volume is brought to 10 ml. with distilled water.

Trihydroxyindole reaction

Clean 10 ml. glass-stoppered graduated cylinders are labeled for urine pH 3.1, urine pH 6.7, urine blank, std. 3.1, std. 6.7, and std. blank. One ml. of the acid eluate from the urine is placed in each tube labeled urine and 1 ml. of the standard solution is placed in each std. tube.

Two ml. of acetate buffer pH 6.7 is added to each tube labeled pH 6.7 and to each blank.

Two ml. of acetate buffer pH 3.1 is added to each pH 3.1 tube.

Mix well. Add 0.1 ml. potassium ferricyanide to each tube and 0.2 ml. $ZnSO_4$ solution to each pH 3.1 tube.

Shake well. Exactly 2 minutes after addition of the ferricyanide, add 0.5 ml. of the ascorbic acid - NaOH solution to each pH 6.7 tube.

Exactly 3 minutes following addition of ferricyanide, add 0.5 ml. ascorbic acid-NaOH solution to each pH 3.1 tube.

To the blank tubes, 2 minutes after addition of ferricyanide, add 0.9 ml. of 20 per cent NaOH, let stand 15 minutes, then add 0.1 ml. of ascorbic acid solution.

Add distilled water to each tube to bring the volume to 10 ml. Centrifuge each tube for 1 minute.

Determination of Fluorescence

The fluorescence of the solution in each tube is determined in an Aminco-Bowman Spectrophotofluorometer with an activation setting of 415 millimicrons and a fluorescent setting of 515 millimicrons.

The fluorescent compounds are stable for 10 - 15 minutes. It is suggested that not more than 4 tubes be oxidized and the fluorescence determined at a time.

Calculations (E = epinephrine; NE = norepinephrine)

$$NE = y = \frac{a(A-B)}{s-n}$$

$$E = X = \frac{a(B-F)}{sm} - \frac{ny}{sm}$$

a = conc. of std. solution (10 micrograms per 100 milliliters)
A = urine reading at pH 6.7.
B = urine reading at pH 3.1.

s = standard reading at 6.7 minus standard blank.
n = Percent oxidation of norepinephrine at pH 3.1 $= \frac{\text{NE reading at pH 3.1}}{\text{NE reading at pH 6.7}} \times 100$
F = reading of urine blank.
y = calculated amount of norepinephrine
m = ratio of fluorescent intensity of stand ard solutions (E/NE) of equal concentration at pH 6.7. This ratio will change somewhat with each filter system and must be calculated for each machine. The ratio, once determined, will remain constant for the machine.

Recovery of added NE from urine

Ten micrograms of NE were added to urine of which 90 per cent was recovered.

Specificity

Epinephrine, norepinephrine, isoprenaline and to a limited extent pyrogallol produced fluorescence with the trihydroxyindole method. The activation and emission wave lengths of these compounds, except pyrogallol, were reported previously.[8] The activation and emission wave lengths of pyrogallol are 450 millimicrons and 500 millimicrons respectively. Excitation of pyrogallol at 415 millimicrons, as for E and NE, produces a weak fluorescence at 515 millimicrons, the intensity of which corresponds to 6 per cent of the intensity of E.

Discussion

While working on urinary catechol amines, by the method of von Euler and Floding, the investigator noticed that the intensity of fluorescence at pH 3.1 was higher than at pH 6.7 in some urine specimens. This abnormal finding may suggest the existence of some interfering substances in the urine since the fluorescent intensity of a mixture of E and NE is always less at pH 3.1 than at pH 6.7.

Several organic solvents to eliminate interfering substances were tried. None of these proved successful but n-butanol was found to be a suitable solvent for this purpose. Epinephrine and norepinephrine cannot be removed by n-butanol but remained in the acid part.

The centrifugation of the final solution, just before fluorescence is determined, is another factor in the elimination of interfering substances. Butanol washing can remove a great part of these compounds but there will still remain some interfering substances which form a cloudy, reticular zone, after centrifugation, at the bottom of the tube. The intensity of fluorescence of the reticular part is very strong, so that high fluorescence can be found in uncentrifuged solutions (see table 1).

The method of von Euler and Floding is a reliable procedure in physiological studies and in the diagnosis of pheochromocytoma. But in some instances urine contains interfering substances which give reactions with the trihydroxyindole method and can lead to

incorrect results. With the method of von Euler and Floding it is not possible to get rid of these compounds but with the modified method the interfering substances ceased to be a problem.

Normal ranges for urinary E and NE in our laboratory are 1-15 micrograms per 24 hours and .10-60 micrograms per 24 hours respectively.

TABLE 1

THE EFFECT OF BUTANOL WASHING ON INTERFERING SUBSTANCES IN URINE

Urine Specimens	Urine extract not washed with butanol, fluorometric readings at pH			Urine extract washed with butanol, flurometric readings at pH			Solution of the reticular zone, fluorometric readings at pH		
	3.1	6.7	Blank	3.1	6.7	Blank	3.1.	6.7	Blank
1 —	425	200	70	75	120	55	800	315	190
2 —	180	145	85	65	95	35	310	170	110
3 —	80	150	80	45	90	35	130	225	130

Summary

A modification of the von Euler and Floding method of determining urinary catechol amines is presented.

REFERENCES

1. Lund, A.: Acta Pharmacol. et Toxicol., 7: 309, 1951.
2. Goldfien, A., Zileli, M.Ş., Despointes, R. H., Bethune, J. H.: Endocrinology, 62: 749, 1958.
3. Cannon, W. B., Mc Iver, M.A., Bliss, S. W.: Am. J. Physiol. 69: 46, 1924.
4. Elmadjian, F., Hope, J. M., Lanson, E.: J. Clinical Endocrinol. and Metab. 17: 608, 1957.
5. Havens, L. L., Zileli, M. Ş., Dimascio, A., Boling, L., Goldfien, A.: Biological Psychiatry, San Francisco, May, 1958, Published by Grune and Stratten, New York, London, 1959.
6. Walker, W. F., Zileli, M.Ş., Reutter, W., Shoemaker, W. C., Friend, D. G. Moore, F. D.: Am. J. Physiol. 197:773, 1959.
7. Zileli, M. Ş., Walker, W.F., Reutter, W., Shoemaker, W. C., Friend, D. G.: unpublished data.
8. Zileli, M.Ş., Hamlin, J.T., Reutter, W., Friend, D. G.: J. Clin. Investigations 37: 409, 1958.
9. Valk, A. T., Price, H. L.: J. Clin. Investigations 35:837, 1956.
10. Euler, U. S. von, Floding, I.: Acta Physiol. Scand., 33 Suppl., 118: 45, 1955.