DETECTION OF VITAMIN B_6 DEFICIENCY

Utilization of an Improved Method for Rapid Determination of Xanthurenic Acid in Urine*

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It is well known that a deficiency of Vitamin B_6 (pyridoxine) and related compounds will alter the normal metabolism of tryptophan in such a manner as to lead to the excretion of xanthurenic acid (XA) in the urine. In man, the administration of a test dose of 10 Gm. of dl-tryptophan is followed by a significant excretion of XA in the urine of healthy persons on an experimental vitamin B_6-deficient diet.[2] The application of this procedure can be used for the detection of a vitamin-B_6 deficiency in various clinical conditions. Thus, Sprince and his associates[6] have recently found, after a test dose of tryptophan, a significant increase in the concentration of xanthurenic acid in the morning specimen of urine of women whose pregnancy was complicated by toxemia as compared to normal women with and without pregnancy. By measuring the total amounts of XA following the test dose of tryptophan, we were able to show that a relative deficiency of vitamin B_6 exists, not only in pre-eclamptic pregnancy, but also in women with uncomplicated pregnancy, and that this deficiency is already present at the end of the first trimester.[7]

For the determination of XA we followed essentially the procedure of Rosen, Lowy and Sprince,[5] which eliminates the preliminary time-consuming extraction of XA from the urine prior to its colorimetric determination. It was found, however, that certain simplications could be made that expedite and facilitate the performance of the test with no loss of accuracy. This simplified procedure is the subject of this communication.

MATERIALS AND METHODS

Reagents and apparatus. (1) 0.4 M tris (hydroxymethyl) amino methane buffer pH 7.8.[1] This is prepared by dissolving 58 Gm. of maleic acid (C.P.) and 60.6 Gm. of tris (hydroxymethyl) aminomethane (obtained from Commercial Solvents Corp., New York 17, N. Y.) in 500 ml. of distilled water. About 4 Gm. of charcoal is added. The mixture is then shaken and, after allowing it to stand for 10 minutes, is filtered; 48.4 ml. of 1 N NaOH is added to 40 ml. of the filtrate, and after diluting it to 100 ml. with distilled water the pH is checked with a pH meter and any necessary adjustments are made. The buffer remains stable in the icebox. (2) 1.7 per cent ferric ammonium sulfate solution (0.85 Gm. of $FeNH_4(SO_4)_2 \cdot 12\ H_2O$ analytical-reagent grade dissolved in 50 ml. of water). (3) A solution of 60 mg. of pure xanthurenic acid† in 100 ml. of ethanol. The substance is brought into solution by drop-wise addition of 1 N ammonium

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hydroxide. (4) Leitz-Rouy Photometer with a filter at a wavelength of 610 mμ (any type of colorimeter can be used).

Procedure. Place 2 ml. of filtered urine and 8 ml. of distilled water in a dry test tube without further adjustment of the pH. More urine can be used if only small amounts of XA are present. Add 10 ml. of the buffer and mix by inversion. Transfer 10 ml. to a tube marked "blank" and the remaining 10 ml. to another tube marked "unknown." To the "unknown" add 0.1 ml. of 1.7 per cent solution of ferric ammonium sulfate and shake. After allowing the test solutions to stand for 5 minutes, set the colorimeter with the "blank" at 100, replace the blank with the unknown and read the meter. Determine the XA concentration in

TABLE 1

RECOVERY OF XANTHURENIC ACID (XA) ADDED TO URINE

AMOUNT URINE ASSAYED	URINARY XA	ADDED XA	XA RECOVERED	RECOVERY
ml.	*μg.*	*μg.*	*μg.*	*Per Cent*
4	0	600	594	99
4	0	500	500	100
4	92	250	342	100
4	92	100	192	100
4	2.6	80	81	98
4	2.6	50	47.6	90
4	2.6	25	23.8	85
1	0	600	594	99
1	0	500	500	100
1	8.4	250	258.4	100
1	8.4	100	108.4	100
1	0	80	79.2	99
1	0	50	47.5	95
1	0	25	23	92

mg. per 100 ml. from the table. Urine for later study with toluol added can be kept in the icebox without loss of XA.

Calibration. From the stock solution various dilutions were made with 5 per cent ethanol, and the test was performed as described above. The readings plotted on a millimeter graph-paper against the concentrations yielded a straight line. A table was made for permanent reference. Table 1 shows satisfactory recovery of XA added to the urine.

COMMENT

The most significant modification in the technic as described above is the introduction of a suitable buffer. Rosen and his associates tried several buffers and found them not workable. Thereafter they made use of sodium bicarbonate as suggested by previous workers. However, the interference caused by the formation of precipitates after the addition of sodium bicarbonate was the main reason why Miller and Baumann[3] preferred previous extraction to the direct determination of XA in the urine. With the tris (hydroxymethyl) aminomethane buffer, the reduction of the ferric xanthurenic complex by sodium hydrosulfite and the subsequent oxidation of the reactants are omitted. This

procedure in our hands proved difficult and time-consuming, since exact quantitative measurements of the reagents were necessary. The blank values were obtained by setting urine and buffer mixtures at 100 per cent transmission. The recovery of added amounts of pure XA was excellent, and recovery assays performed repeatedly on different days gave consistent values. For clinical purposes a permanent calibration as carried out in most routine laboratory procedures was considered satisfactory. With the technic presented in this paper the color that developed after the addition of the reagent in pure solutions of XA, as well as in urine, was stable for several hours at room temperature. In repeated tests a close agreement was found between the results of the method described by Rosen and coworkers and the modifications presented here.

Rosen and his coworkers[5] have demonstrated by paper chromatography and studies of absorption spectrum that the green color resulting on the addition of the ferric ion to urine, after a test dose of tryptophan, was specifically caused by XA. Other substances that may give a green color-reaction with iron salts, such as phenyl pyruvic acid, react only at an acid pH, and the color-reaction will fade within a few minutes. XA in man will appear in the urine only after the administration of tryptophan, even in the presence of severe experimental vitamin-B_6 deficiency with clinical symptoms.[4] Substances that interfere with the color development of the green XA ion compound may be present. The faint brown color that one obtains in most normal urines after the addition of the reagent, gives, however, only very low readings. The calculated amount for a 24-hour specimen of these interfering substances prior to the test dose of tryptophan may be subtracted from the total amount of XA excreted following the administration of the amino acid.

SUMMARY

A modification of the rapid assay method for xanthurenic acid in urine as proposed by Rosen, Lowy and Sprince is presented. It makes use of the excellent buffering capacity of tris (hydroxymethyl) aminomethane and of a precalibrated curve for its colorimetric quantitative determinations. The quantitative estimation of the excreted XA in the urine following a test dose of 10 Gm. dl-tryptophan is a simple procedure for the detection of a vitamin-B_6 deficiency.

Addendum. Since this paper was accepted for publication, the recent report of Stix Glazer, H., Mueller, J., Thompson, C., Hawkins, V. R., and Vilter, R. W. (A study of urinary excretion of xanthurenic acid and other tryptophan metabolites in human beings with pyridoxine deficiency induced by desoxypyridoxine. *Arch. Biochem. & Biophys.*, 33: 242–251, 1951) came to our attention. These investigators described still another method for the determination of xanthurenic acid in the urine without previous extraction, using the iron ammonium sulfate reagent for the development of the green iron xanthurenic acid complex.

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