JOURNAL OF NUCLEAR MEDICINE 5:297-301, 1964

Determination of Citric Acid in Serum and Urine Using Br^{82}

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Methods which have commonly been used for determination of citric acid levels in blood and urine depend upon oxidation of citric acid to acetone dicarboxylic acid with subsequent decarboxylation and bromination to pentabromoacetone (1-3). Technics used for quantitation of the pentabromoacetone include photometric determinations with such reagents as pyridine (4), alcoholic sodium iodide (5), thiourea (6), sodium sulfide (7), thiourea-borax-Na_2S reagent (8), formate, molybdate and KI (9) and titrimetric procedures for Br^- released by means of Na_2S (10) or sulfite (11).

The present study proposes a means by which the pentabromoacetone may be measured directly, without the necessity of time-consuming photometric or titrimetric operations, through use of the radioactive isotope of bromine (Br^{82}) in the brominating reagent.

EXPERIMENTAL

Reagents

Perchloric acid, 0.65 M. Mix 5 ml 70 per cent $HClO_4$ with 85 ml distilled water.

Potassium hydroxide, 33 per cent.

Brominating reagent, 2 N: Dissolve 17.16 g NaBr and 5.04 g $NaBrO_3$ in distilled water. Add 10-25 mc Br^{82}, carrier-free (obtainable from Oak Ridge National Laboratory), and dilute to 100 ml with water.

Saturated ferrous sulfate in 0.02 N H_2SO_4.

Sulfuric acid, 12 N.

Potassium permanganate, 5 per cent. Boil for 10 min., cool, filter through glass wool and readjust volume.

Manganese sulfate, monohydrate, 40 per cent.

Hexane, obtained from British Drug Houses, Ltd.

Carbon tetrachloride.

Sodium bromide, 0.01 M. 103 mg per 100 ml distilled water.

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Citric acid stock solution. Dissolve 109.3 mg of citric acid monohydrate in 100 ml 1 N H_2SO_4. This solution is equivalent to 100 mg citric acid per 100 ml and is stable for at least one year

Procedure

Because of the possibility of escape of radioactive bromine gas during the analysis, all operations subsequent to the first addition of brominating reagent should be carried out in a fume hood.

1. To 13.5 ml of perchloric acid reagent add 1.5 ml of serum with continuous mixing; for urine use 1.5 ml of a 1:20 dilution. Wait 10 min and centrifuge. (When abnormal quantities of acetone bodies are suspected, they should be eliminated by the following procedure: Using doubled quantities of materials, 20 ml of the clear supernatant is concentrated to 6 ml by boiling and the volume restored by addition of water.) A citric acid standard solution, 4 mg per cent, which may be desired as an additional control in the method, is prepared by diluting the stock solution 1:25 with water and treating with perchloric acid as described. A blank is also prepared by substituting 1.5 ml of water for the specimen.

2. To 20 x 150 mm test tubes add 12 ml of the clear supernatants and 1.2 ml of 33 per cent KOH. After vigorous mixing, place the tubes in an ice bath for 5 min and separate the $KClO_4$ precipitates by centrifugation. Transfer 10 ml of the supernatants to 35-ml glass-stoppered centrifuge tubes, add 5 ml of 12 N H_2SO_4 with mixing and 1 ml of the radioactive brominating reagent. Stopper, mix and allow bromination to proceed at room temperature.

3. After 30 min remove the Br_2 gas from above the liquid surface with suction, trapping the vapors in dilute sodium hydroxide. To each tube add 2 ml of the ferrous sulfate solution, stopper and shake vigorously. Add 6 ml of carbon tetrachloride, stopper and shake vigorously for 3 min and centrifuge.

4. After centrifugation, transfer 15 ml of the aqueous phase to 50-ml glass-stoppered tubes, cool to 18°C in a water bath and maintain at this temperature for the subsequent oxidation-bromination step. To each tube, add 1 ml of $MnSO_4$ and 1 ml of radioactive brominating reagent. Slowly add 2.5 ml of $KMnO_4$ with mixing; shortly after this a brown MnO_2 precipitate should appear.

5. After incubating in the 18°C water bath for 30 min, remove the Br_2 vapors as described above and add 6 ml of ferrous sulfate reagent. After mixing, extract the radioactive pentabromoacetone by shaking the tube with 10 ml of hexane for 6 min. Centrifuge and transfer duplicate 2-ml aliquots of the hexane phase to test tubes suitable for use with a well scintillation counter. Wash the hexane aliquots by shaking with 5 ml of 0.01 M NaBr, centrifuge and remove the aqueous phase from beneath the hexane using a fine-tipped pipet and suction.

6. Radioassay the hexane aliquots as well as a 2-ml aliquot of a 1:1000 dilution of the brominating reagent for use as the standard in the calculations.

Calculations

All gross sample count rates, before being used in the following calculations, are corrected by subtracting the gross count rate of the blank. The standard is corrected for background radioactivity only.

Let S_c = c/m in the standard equivalent to 1 mg of citric acid (1:1000 dilution of brominating solution, 2 ml.)

U_s = c/m in hexane aliquot from serum analysis.

U_u = c/m in hexane aliquot from urine analysis.

Then $S_c = \frac{\text{c/m in the standard}}{0.160} \times \frac{400}{192}$, where 0.160 is the number of mg of Br in the aliquot of standard and the factor $\frac{400}{192}$ converts the mg Br in pentabromoacetone to the equivalent number of mg of citric acid.

Serum citric acid, mg% $= \frac{U_s}{S_c} \times \frac{100}{0.151}$, where 0.151 is the actual volume of serum in the final assay step.

Urine citric acid, mg% $= \frac{U_u}{S_c} \times \frac{100}{0.0076}$, where 0.0076 is the actual volume of urine in the final assay step.

Study of the Method

The method of Taussky (12), based on conversion of citric acid to pentabromoacetone and the reaction between this and sodium iodide to give a yellow color was employed as the method of reference for comparison with the newly-developed procedure. The only deviation from that of Taussky was substitution of hexane for heptane.

Table I shows the results obtained on five freshly drawn normal sera and six fresh normal urine samples. Recoveries of citric acid added to 2 serums and 2 urines at levels of 5 and 100 mg per cent, respectively, and carried through the isotope procedure, were 96 to 109 per cent (mean, 102%). Precision, calculated from duplicate determinations was ± 9.2 per cent (95% limits) for 5 serum and ± 6.6 per cent for 6 urine assays. Reagent blanks carried through the procedure gave an apparent citric acid concentration of 0.3 to 0.4 mg% per cent for serum and 6.2 to 8.4 mg per cent for urine.

Comparison of the paired results obtained by the two methods revealed no significant difference for serum; for urines, on the other hand, the Br^{82} method resulted in a distinct increase that was highly significant ($P<0.01$).

DISCUSSION

The validity of this method rests on the assumption that the radioassay value represents pentabromoacetone prepared from bromine-82 of known specific activity. The determination of this specific activity is simply achieved through

knowledge of the Br^{80} concentration in this reagent and the subsequent radioassay of an aliquot. This assumption is valid so long as no other Br^{80} is admitted to the procedure. It is for this reason that the first bromination operation (step 2) requires the use of the radioactive reagent since bromine is carried over into the aqueous phase used in step 4, the preparation of pentabromacetone from citric acid.

Another assumption is that no other radioactive materials than pentabromoacetone contribute to the radioassay value. In this connection, two serious problems arose with respect to reagents. It was discovered that trichloroacetic acid and hexane, or impurities in these reagents, became labelled with Br^{82}, thus producing high and erratic blanks. Substitution of perchloric acid as protein precipitant in place of the trichloroacetic acid and substitution of carbon tetrachloride for hexane in the first solvent extraction step eliminated these problems. Hexane could still be used for the final extraction since there is no opportunity for bromination of the final extractant.

Comparison of the Br^{82} and photometric methods for the assay of serum (Table I) reveals that there is no significant difference between the two methods. In the case of urines, however, the Br^{82} technic yields results averaging 11 per cent higher than does the photometric method. The reason for this is not readily

TABLE I

COMPARISON OF RADIOMETRIC AND PHOTOMETRIC METHODS

Sample	*Citric acid concentration, mg%*	
	Br^{82} Method	*Photometric Method*
Serum		
1	2.61	2.46
2	2.55	2.69
3	1.89	1.77
4	2.57	2.50
5	2.55	2.55
Urine		
1	42.4	39.0
2	24.6	23.0
3	63.6	58.5
4	56.8	49.8
5	29.5	25.5
6	9.2	8.2

apparent, although it would seem that there may be some substance (or substances) in urine which upon oxidation and bromination yields a derivative which is stable toward alcoholic sodium iodide so as not to be determined in the photometric procedure.

The proposed method is of particular value when a large number of samples are to be run together. Time-consuming washing of hexane extracts, and preparation of the unstable iodide reagent, setting up of color reaction and photometry itself are eliminated from the determination. For this is substituted the much simpler and readily automated counting procedure. Moreover, problems often encountered in the photometric technic due to insufficient purification of the solvent hexane are not present here.

SUMMARY

Radiometric assay for Br^{82} was used in the analysis for citric acid by incorporation of this isotope in the brominating reagent for conversion of the citric acid to pentabromoacetone. This provided a rapid and simple means for quantitative recovery of added citric acid and a precision (95% confidence limits) of $\pm$ 9.2 per cent and $\pm$ 6.6 per cent was obtained for serum and urine specimens, respectively. Results by this technic were slightly higher for urine than those obtained by the photometric method used for comparison, whereas results for serum agreed well.

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