Ultra-micro Sugar Determinations Using 2,9-dimethyl-1,10-phenanthroline Hydrochloride (Neocuproine)

Mayo E. Brown, M.S., Boston

This communication is concerned with a chemical method for the determination of blood sugar by an "ultra" micro technic on blood volumes as little as 0.01 to 0.03 ml. The need for such a method and its development occurred during experiments on small diabetic animals (guinea pigs) when difficulties were encountered in collecting as little as 0.05 to 0.1 ml. of blood. Although the literature abounds in reports on technics for micro blood sugar determinations, no copper reduction method has yet been reported to be applicable to these small quantities of blood.[1-5,14]

This method follows that originally described by Folin and Wu, with two important modifications, namely, a twentyfold reduction in the amount of crystalline copper sulfate used to prepare the alkaline copper solution, and a replacement of the phosphomolybdic acid by a solution of neocuproine.

The use of 2,2′-diquinoline (cuproine) for the detection of the cuprous ion was described by Hoste in 1950.[6] Later reports by Smith, Gahler and others showed that 2,9-dimethyl-1,10-phenanthroline (neocuproine) was highly specific for the cuprous ion, and that the presence of fifty-six other metallic ions, including the cupric ion, did not interfere in the reaction of neocuproine with cuprous ion.[7-9] It was further shown that the final color was very stable over a *p*H range of 3 to 10, and that the presence of chloride, tartrate, citrate, acetate, phosphate and several other negative radicals did not interfere with the final color development. The availability of the water soluble neocuproine hydrochloride salt suggested that it might serve as a replacement for the various "molybdic acids" used in the copper reduction sugar methods.

In order to validate the use of an "ultra" micro blood sugar technic, comparison with the macro Folin-Wu blood sugar method was made.

Reagents:

(A) Alkaline dilute copper solution.
Dissolve 40 gm. of anhydrous sodium carbonate in about 400 ml. of distilled water in a liter flask. Add 7.5 gm. of tartaric acid and, when dissolved, add 0.225 gm. of crystalline copper sulfate ($CuSO_4 \cdot 5H_2O$). Mix and dilute to the liter mark. (This solution contains about 58 μg. of copper per milliliter.) Alternately, the Folin-Wu alkaline copper solution may be diluted twentyfold with a solution containing 40 gm. of sodium carbonate and 7.5 gm. of tartaric acid per liter.

(B) 0.4 neocuproine solution.
Dissolve 0.4 gm. of neocuproine hydrochloride* in 100 ml. of distilled water. This solution is colorless and stable for six months or longer, provided it does not become contaminated with cuprous ions.

(C) 0.0417 (1/24) normal sulfuric acid.
Dilute 1/12 normal sulfuric acid (as proposed by Haden)[10] with an equal volume of water.

(D) 10 per cent sodium tungstate W/V.

Preparation of 1:101 blood filtrate:

The blood sample 0.02 ml. (20 lambda) is added to 1.95 ml. of 0.0417 *N* sulfuric acid with careful rinsing of the blood pipette,† and immediate shaking of the tube. Then 0.05 ml. of 10 per cent sodium tungstate is added, mixed, and allowed to stand for ten minutes. Upon centrifugation a clear supernatant solution is obtained.

Procedure:

1. One milliliter of 1:101 filtrate (or any equivalent amount of solution containing from 5 to 30 μg. of glucose) is placed in a 10-ml. Sunderman sugar tube.‡ (See footnote on page 61.) Volumes other than 1 ml. are made up to 1 ml. with distilled water.

2. A blank of distilled water and glucose standards (10, 20 and 30 μg.) are similarly prepared.

From the Laboratory of The Faulkner Hospital, Boston, Massachusetts.

*The neocuproine hydrochloride (2,9-dimethyl-1,10-phenanthroline hydrochloride) was obtained upon request from G. Frederick Smith Chemical Company, Columbus, Ohio.

†A reliable 20 cmm. Sahli hemoglobin pipette, or a 25 lambda pipette calibrated in 5 lambda may be used.

3. Two ml. of the alkaline dilute copper solution A are then added to each tube.

4. Add 0.3 ml. of the 0.4 per cent neocuproine (solution B).

5. The sugar tubes are then placed in a vigorously boiling water bath for four to six minutes. Whatever time is chosen should be adhered to for all of the tubes in the series, including the blank. We have routinely used a time of five minutes.

6. The tubes are removed from the water bath and cooled sufficiently to permit handling, and then diluted to the 10 ml. mark with distilled water. The tubes are mixed by inversion several times.

7. Using a Coleman model 6A spectrophotometer with 19 x 150 mm. cuvettes, the blank is set at zero optical density at 450 mu.

8. The standards are sufficiently reproducible to construct a permanent calibration chart from which to read the unknowns.

Calculations:

According to the above outlined procedure, the glucose standards of 10, 20 and 30 μg. (because of the 1:101 dilution) are equivalent to 101, 202, and 303 mg. of glucose per 100 ml. of whole blood.

RESULTS

Figure 1 (upper curve) illustrates the relationship of the optical densities to various glucose concentrations. The curve is linear from 0 to 30 μg. of glucose. The lower curve of figure 1 for hydroxylamine reduced copper (0 to 40 μg.) shows that 20 μg. of reduced copper is equivalent to 14.6 μg. of glucose. This reduced copper to glucose relationship is predictable by extrapolating the glucose values (equivalent to 0.1 to 4.0 mg. of reduced copper) obtained by Somogyi[11] and others.[12]

Figure 2 illustrates that all of the whole blood sugar values obtained by the method presented agree within 10 per cent with those obtained by the classical Folin-Wu method. In fact, the average error of fifty-one analyses was 5.1 per cent. The coefficient of correlation between these methods was 0.96, as shown in figure 2. The error of duplicate analysis by the proposed method was less than 5 per cent, which is about the same as that found for duplicate macro Folin-Wu sugar analyses.[12] Duplicate analyses of the glucose standards were within 3 per cent.

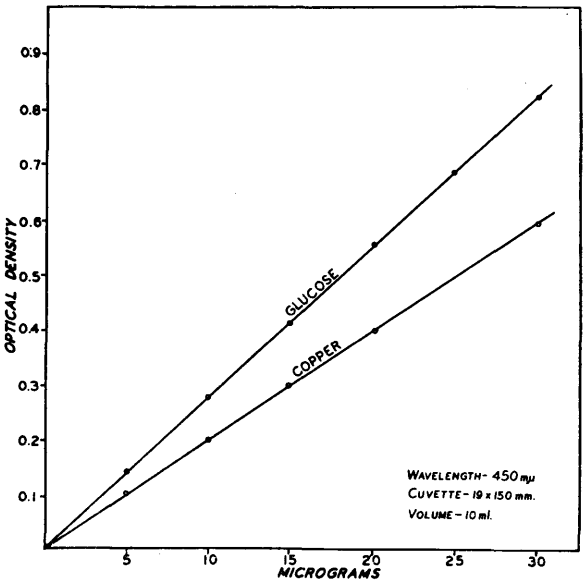

FIG. 1. Illustrates the optical densities obtained for glucose and reduced copper of similar concentrations. The glucose determinations were made according to the procedure described in the text. The copper sulfate standards were reduced with hydroxylamine hydrochloride, and the pH adjusted to 9.8 with an alkaline-tartrate solution before the final cuprous-neocuproine was developed by the addition of the neocuproine solution.

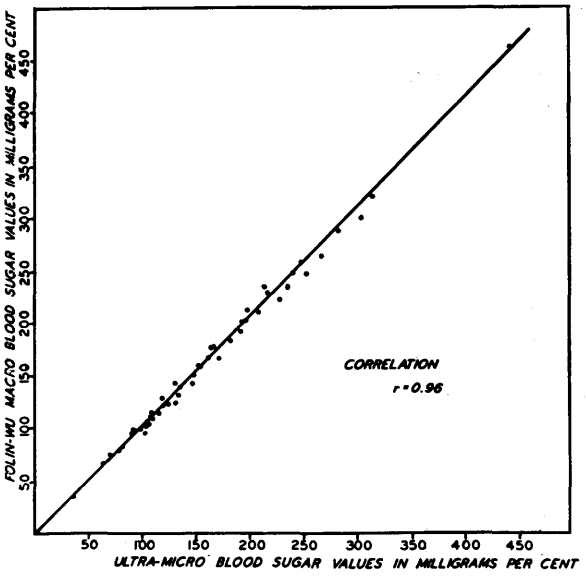

FIG. 2. The sugar values of fifty-one whole blood samples were obtained by the classical macro Folin-Wu method and the ultra-micro neocuproine method as proposed. A significant coefficient of correlation of 0.96 was obtained when the values for the two methods were compared.

‡ Where it is inconvenient to obtain calibrated 10 ml. sugar tubes, the ordinary 25 ml. Folin sugar tube may be used by the addition of 0.7 ml. of distilled water to all the sugar tubes so that the final volumes of the tubes in Step #4 is 4 ml. In Step #6, the addition of 6.0 ml. of distilled water will yield a volume sufficiently close to 10 ml. so that reproducible results are obtained.

Neocuproine could be incorporated into the alkaline dilute copper solution for each day's analyses without adverse effects. The neocuproine solution added to alkaline tartrate solution (containing no copper) and boiled for ten to twenty minutes did not produce color. Sufficient amounts of neocuproine remained to detect several hundred micrograms of reduced copper.

When several sets of 20 μg. glucose standards with their respective blanks were boiled for periods from two to eight minutes, it was found that the three- to eight-minute boiling periods did not alter the amount of copper reduced by more than 2 per cent. The two-minute boiling period of the standard with its respective blank reduced 68 per cent of the theoretical amount of copper. The blanks for the above corresponding boiling periods (three, four, five, six and eight minutes) were found to be equivalent to 3.3, 5.5, 6.8, 7.4 and 7.9 μg. of glucose respectively. While this amount of copper reduction in the blanks was considered slightly more than that expected for the auto-oxidation-reduction of alkaline copper solutions, it nonetheless did not affect the reproducibility of the standards when a blank was included.

When the alkaline dilute copper solution was made to contain varying amounts of copper sulfate (100 to 300 μg. of copper) and treated as a blank according to the procedure, it was found that an average increase equivalent to 0.2 μg. of glucose occurred for every 10 μg. of copper. Thus a 10 per cent error in pipetting the alkaline dilute copper solution would result in less than 2 per cent error.

Changing the final *pH* (9.8) to various *pH* values (5.0 to 10.0) by dilute acid or buffer did not affect the colorimetric readings, but served only to release carbon dioxide and make colorimetric readings more difficult. The final orange-red- neocuproine-cuprous complex was stable for ten hours or more in diffuse sunlight at room temperature.

COMMENTS

The only objection to the use of neocuproine hydrochloride as a color reagent for sugar analyses was that the blanks were slightly greater than anticipated. On the other hand, neocuproine was found to be about thirty times more sensitive to the cuprous ion than Folin's phosphomolybdic acid, and a more careful consideration would reveal that an equivalent amount of auto-oxidation-reduction of the alkaline copper solution also occurs in the classical Folin-Wu method.

The above procedure has been adapted to the Klett and Evelyn colorimeters (440 filters), with slightly higher optical density readings for the glucose standards by the latter instrument. Hence the effective range with the Evelyn colorimeter was from 4 to 20 μg. of glucose. Further reduction of the final reading volume from 10 ml. to 6 ml. permitted determinations on samples containing from 2 to 15 μg. of glucose.

Use of neocuproine in other copper reduction methods (Somogyi, Benedict) tentatively appears feasible.

SUMMARIO IN INTERLINGUA

Ultramicrodeterminationes de Sucro Sanguinee con le Uso de Hydrochloruro de 2,9-Dimethyl-1,10-Phenanthrolina (Neocuproine)

Le presente reporto es concernite con un methodo pro le determination de sucro sanguinee per medio de un ultramicrotechnica que require volumines de sanguine de solmente 0,01 a 0,03 ml. Ben que le litteratura abunda in reportos de technicas pro le microdetermination de sucro sanguinee, nulle methodo a reduction de cupro ha essite reportate con applicabilitate a specimens del micre magnitudes hic considerate.

Le methodo seque illo originalmente describite per Folin e Wu, con duo importante modificationes, i.e. (1) un reduction vintuple in le quantitate de crystallin sulfato de cupro usate in preparar le solution alcalin de cupro e (2) le substitution de un solution de neocuproina pro acido phosphomolybdic.

REFERENCES

[1] Morris, D. L.: Quantitative determination of carbohydrates with Dreywood's anthrone reagent. Science *107*:254, 1948.

[2] Park, J. T., and Johnson, M. J.: A submicro determination of glucose. J. Biol. Chem. *181*:149, 1949.

[3] Lee, J.: A quick and simple method for blood-sugar estimation. Brit. M. J. *2*:1087, 1954.

[4] Caraway, W. T., and Fanger, H.: Ultra micro procedures in clinical chemistry. Am. J. Clin. Path. *25*:317, 1955.

[5] Froesch, E. R., et al.: Specific enzymatic determination of glucose in blood and urine using glucose oxidase. Diabetes *5*:1, 1956.

[6] Hoste, J.: A new copper specific group. Anal. Clin. Acta *4*:23, 1950.

[7] Smith, G. F., and McCurdy, W. H., Jr.: 2,9-dimethyl-1,10 phenanthroline, new specific in spectrophotometric determination of copper. Anal. Chem. *24*:371, 1952.

[8] Gahler, A. R.: Colorimetric determination of copper and neocuproine. Anal. Chem. *26*:577, 1954.

[9] Luke, C. L., and Campbell, M. E.: Determination of impurities in germanium and silicon. Anal. Chem. *25*:1588, 1953.

[10] Haden, R. L.: A modification of the Folin-Wu method for making protein-free blood filtrates. J. Biol. Chem. *56*:469.

[11] Somogyi, M.: New sugar reagent. J. Biol. Chem. *160*:61.

[12] Shaffer, P. A., and Hartman, A. F.: The iodometric determination of copper and its use in sugar analysis. J. Biol. Chem. *45*:365, 1920-21.

[13] Host, H., and Hatlehal, R.: Blood sugar concentration and blood sugar methods. J. Biol. Chem. *42*:347, 1920.

[14] Cauley, L. P., et al.: Ultramicrochemical analysis of blood glucose with glucose oxidase. Am. J. Clin. Path. *29*:111, 1959.