AN ULTRAMICRO-COLORIMETRIC METHOD FOR THE DETERMINATION OF PLASMA GLUCOSE WITH O-AMINOBIPHENYL, CIRCUMVENTING DEPROTEINZATION

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Hagedorn-Jensen's ferricyanide iodometric method[1] and Somogyi-Nelsons, copper sulfate-arsenomolybdate colorimetric method[1] have been the most favored for blood glucose determination in clinical laboratories of this country. However, the procedures are too complicated for routine work, involving three steps of (1) deproteinization, (2) reduction and (3) end point reaction for titration or colorimetry.

Recently, a direct method to determine plasma glucose with glucose oxidase and peroxidase has been reported from the United States[2]. However, these enzymes with high potencies are hard to obtain at present, and it has been hoped in our laboratory that another one-step method using common reagents be developed. Orthoaminobiphenyl, a reagent discovered and recommended by Timell[3] several years ago for the detection of carbohydrate, was studied from this view point. When heated with aldopentoses, aldohexoses, methyl aldopentose, hexuronic acids and some oligosacharides, o-aminobiphenyl in glacial acetic acid produces yellowish brown or yellowish green color. Athanail and Cabaud[4] devised a method for blood glucose utilizing this color reaction in combination with deproteinization. The procedure to be presented in this paper is the result of our attempt to develop a direct method for plasma (or serum) glucose using o-aminobiphenyl[5, 6]. The established method has enabled us to omit deproteinization and determine glucose in as little as 0.05 ml of serum with results comparable to those obtained with Somogyi-Nelson's technique.

METHOD

Reagent

1) Stock glucose solution (1000 mg/dl): 1000 mg of pure glucose (Merck) are dissolved in distilled water saturated with benzoic acid in a 100 ml volumetric flask. Aliquots of 0.25, 0.5, 1.0, 2.0 and 4.0 ml of this solution are diluted to 10.0 ml with distilled water to prepare standard solutions of 25, 50, 100, 200 and 400 mg/dl. These solutions can be stored for a week in a refrigerator.

2) O-aminobiphenyl solution (0.5g/dl): 1g of crystalline o-aminobiphenyl is dissolved in 200 ml of purified glacial acetic acid, and kept in a dark place for two days befor use. This solution can be used for the subsequent seven days.

3) Boric acid solution: 6 g of boric acid are put into 100 ml of distilled water, allowed to stand overnight, filtered with filter paper. This is a saturated aqueous solution of boric acid.

Purification of glacial acetic acid.

About 1.5 *l* of glacial acetic acid (reagent grade) and 6 g of anhydrous chromic acid are introduced into a 2 *l* flask, and the flask is boiled in a oil bath for 30 minutes with a reflux condenser attached to the top. After cooling, a column still is changed for the reflux condenser. The flask is again heated in the oil bath (in order to distill) at temperature not exceeding 118 C. Another distillation is required to collect the distillate at the boiling point of 114 to 118 C. The yield is about 80% and this purified glacial acetic acid is suitable for the determination.

Procedure

1) Color reaction: With an ultramicropipet place 0.05 ml aliquots of 25, 50, 100, 200 and 400 mg/dl glucose solutions into five 15 × 120 mm tubes. They serve for the calibration curve. Using the same pipet which has been cleaned with distilled water, pipet 0.05 ml of serum or plasma sample into another tube. Add 0.6 ml of saturated boric acid solution and 4.2 ml of o-aminobiphenyl reagent to all the tubes, shake, and heat tubes for 30 minutes in a boiling water bath. A green color will develop.

2) Colorimetry: Remove the tubes from the boiling water bath and cool them in running water for 20 to 30 minutes. Measure the opitical densities in a Klett-Summerson photoelectric colorimeter with a red filter (# 66). Use purified glacial acetic acid alone for setting zero reading.

3) Calibration and calculation: Construct the standard calibration curve by plotting the optical densities against glucose concentrations. Read the glucose concentration of the sample from the optical density and the calibration curve. The value directly refers to the glucose concentration in the serum or plasma in mg/dl.

DISCUSSION

Addition of glacial acetic acid to serum results in coagulation of protein and therefore turbidity. Hence, glacial acetic acid solution of o-aminobiphenyl when added to serum produces a turbidity and the turbidity is increased by heating. However, this obstacle is completely obliterated by diluting serum with water before o-aminobiphenyl solution is added. Addition of 0.6 ml of saturated aqueous boric acid solution to 0.05 ml of serum, as described in the procedure, suits this purpose. Boric

acid in water intensifies the color approximately 1.5 fold as compared with water alone.

Clear color is a prerequiste to colorimetry, but completeness of color development without interferce by concomitant substances is just as important. Large amounts of protein suppress the coloration in this reaction. The concentration of protein in a mixture of serum and o-aminobiphenyl reagent should therefore be adjusted to the level where there is no more intereference. Yet, improper diminution of the amount of serum out of proportion to o-aminobiphenyl reagent attenuates the color. Furthermore, the concentration of o-aminobiphenyl reagent can not be ignored for accurate determination. This reagent fails to give a reliable calibration curve in concentrations less than 0.4 g/dl under these conditions, while it entails shortening of the rectilinear calibration greater than 0.6 g/dl. In our experience, addition of 0.5 g/dl o-aminobiphenyl reagent to serum in a volume ratio of 80:1 or more has been satisfactory. Careful study of these factors has led to the present procedure which specifies that 4.2 ml of o-aminobiphenyl reagent be used for 0.05 ml of serum in combination with 0.6 ml of saturated boric acid solution. Although turbidity is seldom encountered, if it happeus, 4.4 ml of o-aminobiphenyl reagent instead of 4.2 ml is recommended.

The hue of the color varies to some extent with the quality of glacial acetic acid used. While glacial acetic acid of reagent grade is not suitable, that of CP grade has been found satisfactory. If the latter is purified further with chromic acid, the coloration is more ideal.

The absorption spectrum of the color obtained with 200 mg/dl glucose solution using the present procedure is shown in Figure 1. There are two absorption peaks, a higher one at 650 mμ and a lower at 380 mμ. The curve may give an idea that reading at 380 mμ will be more sensitive and therefore suitable than at 650 mμ. However, the absorbance at 380 mμ is not very specific to glucose, since there is significant absorption by serum proteins in that region of wave length. Therefore, light of approximately 650 mμ is preferred, and the use of Klett-Summerson's red filter (# 66) is very practical. Determination at 650 mμ gives a calibration curve which is straight from 0 to 200 mg/dl of glucose with slight bending beyond 200 mg/dl. The line passes a point which is very close to the origin (Figure 2).

The color obtained with icteric serum is different because of the presence of bilirubin which produces bluish color (biliverdin) when heated with glacial acetic acid. Errors due to biliverdin can be eliminated by setting up a blank tube which is prepared by using 4.2 ml of galcial acetic in place of o-aminobiphenyl reagent in the procedure. The absorbance of the sample, s is corrected by subtracting β which is the absorbance of this blank. Thus glucose concentration should be determined from the absorbance s-β. This correction is not feasible with aged sera which have been kept in a refrigerator for more than overnight, since bilirubin is not converted

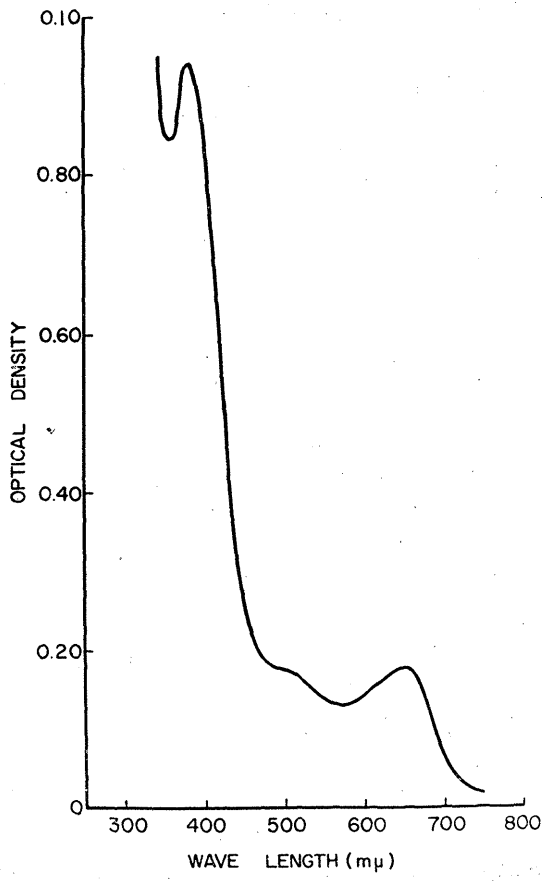

Figure 1. Absorption curve of colored solution (200 mg/dl glucose solution)

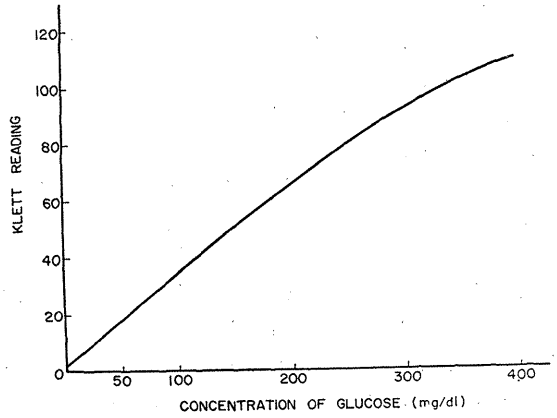

Figure 2. Calibration curve

into biliverdin as readily as in fresh sample with resultant higher values. No blank is necessary, however, for slightly icteric sera (bilirubin lass than 5 mg/dl) or unless excessive hemolysis is present, because neither small amounts of bilirubin nor fairely large amounts of hemoglobin interfere with determination at 650 mμ.

A comparative study with about 100 samples of non-icteric and icteric sera revealed a satisfactory agreement between the results obtained with present procedure and those with Somogyi-Nelson's method (Fig. 3). The correlation coefficient was 0.961 for glucose concentration up to 200 mg/dl and 0.916 for 200 to 500 mg/dl.

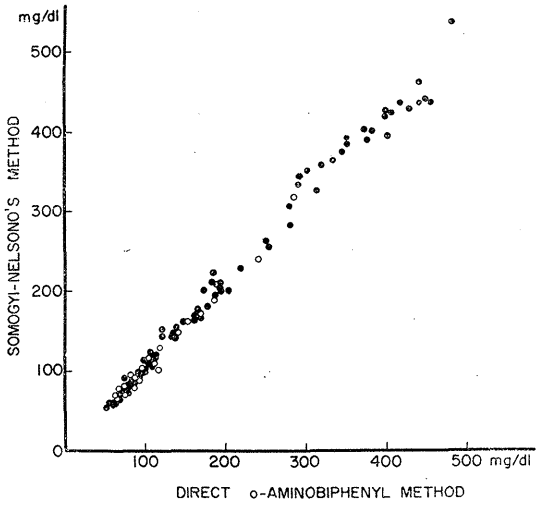

Figure 3. Correlation of o-aminobiphenyl method to Somogyi-Nelson's procedure.

The recovery was also satisfactory, being 99.02±4.9% for a rise in glucose concentration by 100 mg/dl (addition of 0.05 mg glucose to 0.05 ml serum), 96.0± 1.4% for a rise of 200 mg/dl and 88.5±5.95% for a rise of 300 mg/dl.

Complete agreement in duplicate determination is common. Simultaneous determination of eight aliquots from a same sample yielded eight exactly same values. The present method is therefore superior to Somogyi-Nelson's method in precision, and our experience with it as a routine procedure for the past two years has been excellent. This o-amnobiphenyl method can be applied to glucose in spinal fluid without any modification.

SUMMARY AND CONCLUSION

A new direct method using o-aminobiphenyl reagent for the determination of glucose in serum or plasma has been described. To 0.05 ml of serum are added 0.6

ml of saturated boric acid solution and 4.2 ml (sometimes 4.4 ml) of 0.5 g/dl o-aminobiphenyl dissolved in glacial acetic. acid The mixture is heated in boiling water bath for 30 minutes. The resultant bluish green color is measured for absornace in a photoelectric colorimeter at 650 mμ. No deproteinization is required. The values thus obtained compare quite well with those obtained with Somogyi-Nelson's method.

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