A Simple Ultramicro Method for Determination of Serum Galactose Using O-Toluidine Boric Acid

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One of the authors introduced the O-Toluidine Boric Acid Method as a procedure for quantitative determination of glucose about 6 years ago.[1] During the course of review of this method, it was noted that o-toluidine produced specific coloration with hexoses such as glucose, mannose and galactose, and that the effect with galactose was particularly intense being about 1.5 times greater than the former two. Therefore, consideration was of the application of this reaction to the quantitative determination of galactose. Glucose, which would interfere with such a determination, is decomposed by glucose oxidase (there is very little mannose in blood), and efforts were made to develop a simple and quick quantitative determination method by color production of galactose.

A method for the determination of galactose using o-toluidine has been devised,[2] and recently one using galactose oxidase was reported[3] for which kits were developed.[4] However, these methods need 0.2 or 0.4 ml of the sample and require procedures for deproteinization.

By use of a procedure for regulating the concentration of glacial acetic acid in o-toluidine boric acid reagent as in the method for glucose[1] and by carefull checking the conditions of glucose oxidase action, the authors were able to eliminate the deproteinization step. Determination can be made with 20 λ of serum, and the procedure can be applied to the detection of galactosemia in the field of pediatrics and used as a galactose tolerance test to determine the state of liver function.

REAGENTS

1) **1/15 M Phosphate buffer (pH 7.0):** 3.63 g of KH_2PO_4 (Special Grade) and 14.33 g of $Na_2HPO_4 \cdot 12H_2O$ are dissolved in water to make 1000 ml of solution which should be stored at 4° C.

2) **1 g/dl glucose oxidase solution:** 100 mg of glucose oxidase (Tokyo Kasei Co. 1500 u/g) is dissolved in 10 ml of 1/15 M phosphate buffer. Two or three drops of chloroform are added and the solution is filtered through gauze after which it is centrifuged. The supernate fluid is collected and kept at 4°C or frozen (−20°C). In these states it will remain stable for more than 2 weeks.

3) **Saturated boric acid solution:** 6 g of boric acid (Special Grade) is dissolved in 100 ml of water and allowed to stand over-night, after which it is filtered.

4) **O-TB reagent:** 1.5 g of thiourea is dissolved in 890 ml of glacial acetic acid (Special Grade) into which 70 ml of thiourea-o-toluidine solution (Katayama Kagaku Co.) is mixed and 40 ml of saturated boric acid solution are added. This solution is stored in a brown glass container (Effective for at least several months when preserved at 4°C).

5) **0.25 g/dl Benzoic acid solution:** 0.5 g of benzoic acid (Special Grade) is dissolved into 200 ml of water.

6) **Galactose standard solution:** After placing galactose anhydride $C_6H_{12}O_6$ (Katayama Special Grade) in a desiccator for 2–3 days, 200 mg are dissolved into 100 ml of saturated benzoic acid solution. With the benzoic acid solution, the 200 mg/dl galactose solution is diluted to make standard solutions of 0, 50, 100, 150 and 200 mg/dl for calibration. This solution is stable for at least several months.

PROCEDURE

Galactose standard solutions for calibration in concentrations of 0, 50, 100, 150 and 200 mg/dl and sample serum are pipetted in test tubes (15×75) A, B, C, D, E and S in volumes of 20 λ each, to which 0.2 ml of glucose oxidase solution is added. The mixture is incubated to 37°C for 60 minutes to decompose glucose. After enzyme action, 2.0 ml of O-TB reagent is added to each test tube and they are heated to 100°C for 8 minutes using a double walled heating bath.[5] The tubes are then removed from the bath at the prescribed time and cooled with running water. Test tube A is used for O setting at 635 mμ and the optical density for test tubes B, C, D, E and S are read, and a calibration curve is plotted using the optical density readings for tubes B through E. The reading for tube S is plotted and the value is read. This is the concentration of galactose sought.

Galactose of low concentration can be determined merely by increasing the volumes of standard solutions and serum to 50 λ.

RESULTS AND CONSIDERATIONS

A) **Review of conditions for use of glucose oxidase**

In the application of this method, it is of prime importance to completely remove glucose in the serum. Therefore, conditions were chosen to obtain maximum activity of glucose oxidase.

1) **Optimum pH:** 1/15 M phosphate buffer solutions with pH ranging from 4.5 to 8.5 were prepared to determine the optimum pH for glucose oxidase (1500 u/g, Tokyo Kasei Co.) activity. As shown in Figure 1, maximum activity was noted in the vicinity of pH 7.2.

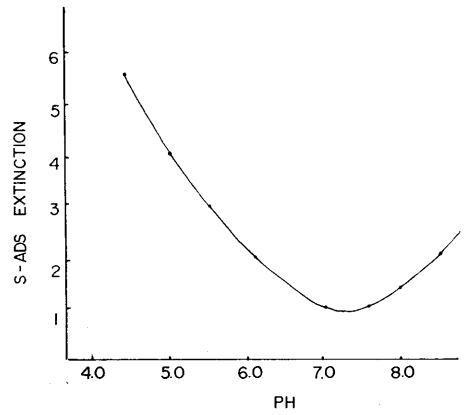

Fig. 1. Optimum pH of Glucoseoxidase

20 λ of 400 mg/dl glucose solution was added to 0.2 ml of glucoseoxidase solution (1500 u) and amount of glucose remaining was determined by coloration of glucose following enzyme action (37°C).

2) **Concentration, temperature and time for enzyme activity:** 1/15M phosphate buffer (pH 7.2) was used to prepare glucose oxidase solutions of 0.25, 0.5, 0.75 and 1.0 g/dl, and 20 λ of glucose solution in concentrations of 100–400 mg/dl were added to 0.2 ml of each of the above oxidase solutions. Enzyme action was allowed to take place for 30 minutes at temperatures of 25°C and 37°C, and the decomposition rates were ascertained (Table 1). It was noted that the decomposition rate was better at 37°C than at 25°C, and was higher as the enzyme concentration increased. When concentrations higher than 0.75 mg/dl are use, 99.5 % of the glucose in patients with values as high as 400 mg/dl can be decomposed. Therefore, practically speaking the glucose will not affect the galactose determination.

On the other hand, the effects of time on enzyme activity are as shown in Figure 2. When glucose oxidase in a concentration of 1.0 g/dl is used and the solution is incubated at 37°C for 30 minutes, 99.6 % of the glucose is decomposed,

Table 1. Rate of Glucose Decomposition by Glucose Oxidase (%)

—— At 25°C ——

Glucose Concentration (mg/dl)	Concentration of Added Glucose Oxidase (g/dl)			
	0.25	0.50	0.75	1.00
100	90.6	96.8	97.1	98.1
200	90.2	96.1	97.5	97.7
300	89.6	95.7	96.8	97.1
400	88.7	94.0	95.5	96.1

—— At 37°C ——

Glucose Concentration (mg/dl)	Concentration of Added Glucose Oxidase (g/dl)			
	0.25	0.50	0.75	1.00
100	97.1	100.0	100.0	100.0
200	96.6	99.6	100.0	100.0
300	96.4	99.5	99.6	99.8
400	95.4	98.9	99.5	99.6

and complete decomposition is achieved when incubated for 60 minutes. Therefore, in the authors' method for glucose decomposition, glucose oxidase in a concentration of 1.0 g/dl is used and the solution is incubated at 37°C for 1 hour.

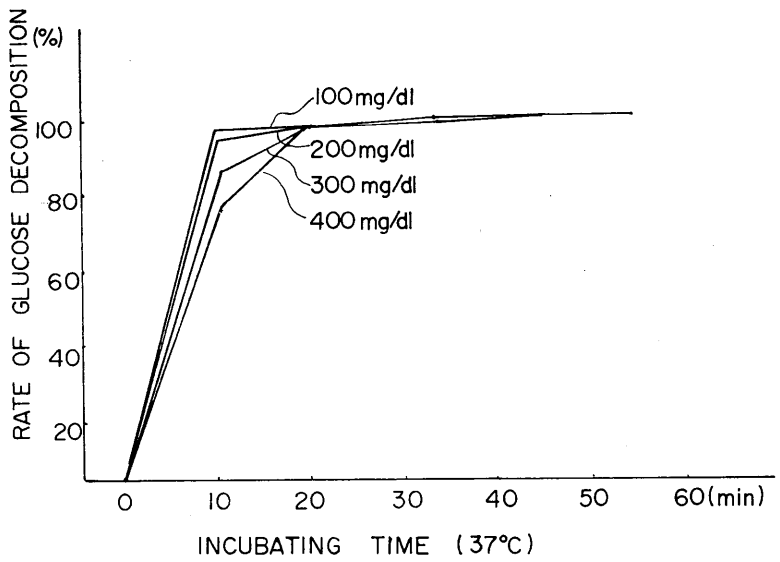

Fig. 2. Decomposition Rate of Glucose by Incubating Time

3) **Specificity of glucose oxidase:** Study was made to determine whether or not glucose oxidase would decompose galactose. The degree of decomposition varied by the enzyme purchased on the market. That is, when 50,000 u glucose oxidase purchased from Sigma Company was used, the result was 8 % at 60 minutes while it was 10.9 % for Deoxine (Nagase Sangyo Co.) and 2.6 % for the presented enzyme. When consideration is given to the fact that it is easier to refine sugar than enzyme, it may be reasoned that the phenomenon described above was due to the difference in purity or titer of enzyme rather than glucose having become mixed with the galactose. Therefore, if glucose oxidase is always added to both the galactose standard solution and the sample and both are subjected to the same processing, it will be possible to correct the degree of impurity to some extent. The presence of benzoic acid does not inhibit enzymic action.

4) **Preservation of aqueous solutions:** It has been pointed out that glucose oxidase in solution is unstable and oxidation and decomposition are promoted by light. However, when kept in an ice chamber or in frozen state, it may be kept for 1 week.[6] Experiments by the authors showed that when the solution is preserved immediately at a temperature below 4° C there was no decrease in titer for more than 2 weeks.

B) **Study of methods of determination**

1) **Absorption curve and calibration curve:** The coloration produced by this method is blue and the absorption curve is shown in Figure 3 with the peak for both galactose and glucose appearing at 640 mμ. In the review of glucose quantitation using O-TB solution reported earlier,[1] the coloration of glucose produced by o-toluidine reached a peak at 635 mμ, but under this experiment it

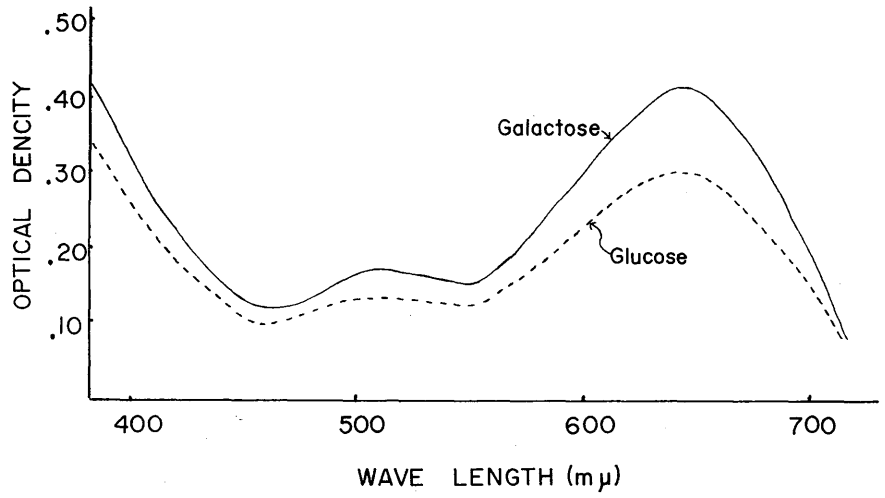

Fig. 3. Absorption Curve
(Hitachi Auto-recording Spectrocolorimeter ····· Model EPS-3T)

was shifted slightly to the infrared. This may be due to the difference in the instrument used for determination, or as pointed out by T. Sasaki,[7] it may be due to the recent marked improvement in purity of o-toluidine reagent as compared to that available at the time of the previous report.

At the wave length of 635 mμ, the calibration curve demonstrated a linear relationship up to 200 mg/dl passing through the point of origin.

2) **Stability and accuracy of coloration:** The greatest defect in coloration by o-toluidine complex is the fading of color. In the experiment performed by the authors, there was 2.2 % fading in 30 minutes at room temperature (20°C) with a wave length of 635 mμ and 6.6 % in 60 minutes. However, as in the case of glucose determination, if colorimetry is performed within 30 minutes after color development, the method is adequate enough for use as a routine test. A recovery test was performed with consideration made for fading. Equal amounts of 100, 200, 300 and 400 mg/dl galactose solutions were added to serum, and the recovery rates were extremely good being 99.2, 99.2, 98.8 and 98.2 %, respectively. Further, 20 tubes of the same serum into which galactose had been added were measured at the same time and the results were 98.5±S.D.* 1.4 mg/dl with CV** = 1.4 %.

3) **Specificity of this method:** The effects of other sugars on method were studied and the results shown in Figure 4. Mannose and Lactose demonstrated a color production rate of 70 and 28 %, respectively, but all of the other sugar showed less than 10 %. As there was difficulty experienced in obtaining galactose oxidase, comparison with the galactose oxidase method could not be made.

Sugars	Reagent Manufacturer	Value converted into terms of Galactose (%)
D-Galactose	Katayama Co. (S.G)	100
D-Mannose	Wako Co. (S.G)	70
D-Glucose	Merck Co. (S.G)	0
D-Fluctose	Wako Co. (S.G)	4
D-Xylose	Wako Co. (S.G)	7
Lactose	Katayama Co (S.G)	28
D-Maltose	Wako Co. (S.G)	9.5
D-Arabinose	Wako Co. (S.G)	7
Starch	Wako Co. (S.G)	0.5
Glycogen	Wako Co. (S.G)	2
Raffinose	Wako Co. (S.G)	3

Fig. 4. Effects of Other Sugars on this Method
····· 100 mg/dl each in aqueous solution was used ·····

* S.D. (Standard Deviation) $= \sqrt{\frac{\Sigma X^2 - \frac{(\Sigma X)^2}{N}}{N-1}}$

**CV (Coefficient of Variation) $= \frac{S.D.}{\bar{X}} \times 100$ (%)

4) **Others:** Review was made of changes in galactose value due to the elapse of time after blood collection and effects of addition of NaF.

When blood was left standing for 6 hours at room temperature after collection, a decrease of 12 % in galactose was noted. This was felt to be due to the so-called "Schwartz test" which is a conversion of galctose to glucose-1-phosphate caused by the actions of G-1-P Uridyl transferase in erythrocytes.[8] As a result, reaction with O-TB reagent does not occur, which brings about decrease of galactose (Study was not made at this time to ascertain whether there was involvement of cells other than erythrocytes. At the same time, a similar experiment was carried out on blood to which NaF had been added, and an 8 % decrease was observed which indicated that effects due to the addition of NaF were not marked. However, as there are no effets of NaF on coloration under this method, it is possible to carry out determinations even on blood to which NaF had been added for other purposes. As in the case of glucose determination, it is necessary to separate the blood cells as soon as possible after drawing of blood. It has been confirmed that the galactose in serum is stable for a long period of time when preserved by freezing.

Other factors such as reactions between o-toluidine and aldohexoses were practically the same as those reported by other workers.[9][10][11]

c) **Normal values**

Early morning fasting state determinations were carried out on a total of 15 male and female laboratory workers whose ages ranged from 18 to 30. The results were 2.5 ± S.D. 0.8 mg/dl. There are surprisingly few reports on the serum galactose values determined during fasting state. Rommel et al[12] reported that it is 10.7 ± 0.8 mg/dl in persons with normal liver function results, and Watson[2] reported that it ranges between 2–10 mg/dl in newborns up to 5 days old.

Fifty hospital patients excluding those with liver function insufficiency and diabetes were selected at random and comparison was made between serum galactose and glucose values during early morning fasting state, but no correlation between the two could be demonstrated (Fig. 5). The degree of variation in galactose values alone was 4.5 ± S.D. 1.6 mg/dl which is slightly higher than those of the healthy persons indicated above.

Review was made also of the galactose tolerance test using this method. The results will be subsequently reported presented in a subsequent report.

CONCLUSION

Review was made of methods to decompose glucose present at time of quantitative determination of serum galactose and to produce color with O-Toluidine

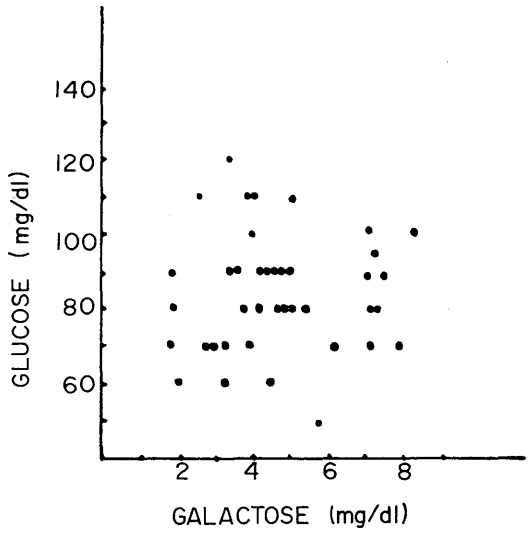

Fig. 5. Comparison of Serum Galactose and Glucose Values in Fasting State (50 cases)

Boric Acid Reagent. The volume of serum required is only 20–50 λ. Therefore, it is possible to make satisfactory determinations even in newborns by using peripheral blood taken from the fingertip. Under this method the calibration curve is linear and passes through the point of origin with the absorption curve reaching its peak value at 635–640 mμ as is also the case for glucose. Adding galactose to test samples showed a recovery rate of 98.2–99.6 %, and the simultaneous determination of 20 tubes of the same seum showed a coefficient of variation of 1.4 %. When compared with the procedure where galactose oxidase is used. This method is inferior in specificity, but is superior from the standpoint of speed, simplicity and economy. It can be used as a routined test for the detection of galactosemia and for the galactose tolerance test for liver function.

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