STANDARDIZATION OF A COLORIMETRIC METHOD FOR THE DETERMINATION OF FRUCTOSE USING O-CRESOL : SULPHURIC ACID REAGENT

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ABSTRACT

Fructose developed a pinkish orange chromogen on treatment with o-cresol : 70% sulphuric acid at 32°C for 15 minutes with a λ max of 500 nm. Fructose was 185 times more chromogenic than glucose. Total carbohydrate and fructose values in protein-free filtrate of normal serum samples were in the range, 55.4-86.3 mg/dl and 1.55-3.29 mg/dl, respectively. In diabetes, the observed values were 197-354 mg/dl and 2.91-6.81 mg/dl, respectively.

KEY WORDS : Fructose determination ; o-cresol : 70% sulphuric acid ; serum values in normals and diabetics.

INTRODUCTION

Several methods for the selective estimation of fructose in biological fluids have been reported (1-5). Most of them are based on the observation that ketohexoses undergo rapid, acid catalyzed dehydration to form hydroxymethyl furaldehyde (6), which condenses with developers to form chromogens. However, a systematic study on the optimal acid concentration, temperature and nature of developers is lacking. Kumudam and Pattabiraman (7) showed that fructose alone reacted with resorcinol and other phenols at room temperature in sulphuric acid medium to form coloured products. o-Cresol and 2,6-dimethylphenol are reported to be better prochromogens in the estimation of hydroxymethyl furaldehyde (HMF) and hexoses than phenol in sulphuric acid medium (8,9). In this communication, we report investigations on acid concentration, temperature, time of reaction and nature of prochromogens ideally suited to selectively estimate fructose in presence of other sugars. A simple, rapid and sensitive method for the estimation of fructose using o-cresol : 70% sulphuric acid at 32°C is de-

scribed. Its application in determination of serum fructose is reported.

MATERIALS AND METHODS

o-Cresol, resorcinol, 2,6-dimethylphenol, fructose, mannose, fucose, ribose, galactose and HMF were the products of Aldrich Chemical Company, Milwaukee, U.S.A. Sulphuric acid used was analar grade (Sp. gravity 1.84) from Ranbaxy Laboratories, Punjab. Other reagents were analytical grade chemicals. Absorbance measurements were made in an Elico spectrophotometer (model SL-159) using cuvettes of one cm pathlength.

DETERMINATION OF TOTAL CARBOHYDRATE

The method described by Rao and Pattabiraman (8) was adapted with the major modification of substituting o-cresol in place of phenol. To one ml of aqueous sugar solution, three ml of concentrated sulphuric acid was added rapidly. The mixture was allowed to stand at room temperature (28-30°C) for 30 minutes. Ethanolic solution of o-cresol (0.1 ml, 10%) was added and mixed. The pinkish orange colour was measured after 30 minutes at 500 nm.

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Chromogens formed from HMF, fructose and glucose absorbed maximally around 495 nm. Calculated molar absorptivities (ϵ) were, 48960 for HMF, 33400 for fructose and 32100 for glucose.

PREPARATION OF PROTEIN-FREE FILTRATE OF SERUM

To 1.9 ml 3.5% trichloroacetic acid, 0.1 ml of serum was added and mixed. After 10 minutes, the sample was centrifuged for 5 minutes in a table centrifuge. Aliquots of the clear supernatant were used for total carbohydrate and fructose estimation.

Roe's resorcinol method was used for determining color intensities formed by fructose and glucose as described by Foremann et al (10). Blood glucose was determined by o-toluidine method (11).

DETERMINATION OF FRUCTOSE

The method developed is as follows. Unless stated otherwise, a mixture of 2.8 ml of concentrated sulphuric acid and 0.8 ml of water was cooled overnight at 4°C. Ethanolic solution (0-1 ml) of o-cresol (10%) was added and mixed. After 2 minutes in a water bath maintained at 32°C, 0.4 ml of aqueous solution containing the sugar was added and mixed. Absorbance was measured at 500 nm after 15 minutes at 32°C. The final acid concentration of the assay medium is 70%.

RESULTS

To attain the different final acid concentrations, 3.6 ml of sulphuric acid : water mixtures (2.4 + 1.2 ml, 2.7 + 0.9 ml, 2.8 + 0.8 ml, 3.0 + 0.6 ml and 3.2 + 0.4 ml) were prepared and cooled. 0.4 ml of sugar solutions were added to get respectively, final acid concentration of 60%, 65%, 70%, 75% and 80%. There was very little chromogen generation below 60% acid level. Chromogens formed with o-cresol, resorcinol and 2,6-dimethylphenol absorbed maximally around 500 nm, 490 nm and 505 nm, respectively. With o-cresol, fructose showed maximal color intensity in the acid range of 70-75%, whereas glucose was maximally active at 75-80%. The ratios of color intensities of fructose and glucose were 330 at 60% acid, 295 at 65%, 185 at 70%, 52 at 75% and 19 at 80% acid concentration. The data indicate that increase in acid concentra-

tion, enhances dehydration of glucose more significantly. The ratios of colour intensities for fructose and glucose with resorcinol at the five acid levels used were, 125, 90, 74, 52 and 31. With 2,6-dimethylphenol, fructose showed sharp activity at 70% acid, whereas glucose showed comparable colour intensity in the range 70-80%. The ratios of color intensities with this developer were 175 (60% acid), 160 (65%), 69 (70%), 44 (75%) and 25 (80%) in favour of fructose. Among the three substituted phenols, o-cresol was found to be best suited in the differential estimation of fructose. Molar absorptivities of fructose at 60%, 65%, 70%, 75% and 80% sulphuric acid in o-cresol reaction were 3000, 12700, 24500, 25200 and 9200 respectively. Even though 2,6-dimethylphenol was as efficient as o-cresol in chromogen yield with fructose ($\epsilon = 24500$ at 70% sulphuric acid), it was less efficient in differential assay. Further, 2,6-dimethylphenol showed a high blank value than o-cresol. In view of these observations, further studies were performed with o-cresol at 70% acid concentration.

With fructose, colour intensity reached 55% of the value seen after 7 hours in 15 minutes. On the other hand, with glucose chromogen formation was almost linear with respect to time upto 3 hours. Thus, lower the time allowed for dehydration, greater is the efficiency of the method in estimating fructose in presence of glucose. In subsequent studies, the time of reaction is fixed at 15 minutes. Fructose developed appreciable color at 25°C and there was gradual increase in intensity with increase in temperature (average $\Delta\epsilon = 840$ per degree centigrade). With glucose, chromogen formation increased rapidly beyond 37°C. Ratio of chromogen formation from fructose and glucose at 45°C (42) was much lower than at 32°C (185). In regard to effect of o-cresol concentration maximum colour yield was obtained at a level of 10 mg of o-cresol. The effect of inclusion of different concentrations of boric acid in the assay system was studied. Boric acid had no effect on colour development in the range, 32-48 mg with fructose. With glucose however, colour intensity increased 2.2-fold at this level. At a concentration of 80 mg., boric acid increased chromogen formation by 29% and 326% for fructose and glucose. Thus, inclusion of boric acid was found to reduce the selectivity of fructose assay.

The colour intensities of fructose and glucose were determined by the resorcinol method (10). The

ratio for the two sugars was 46 and ϵ values for fructose and glucose are respectively, 10000 and 219.

Absorption maxima of the chromogens formed were the same (500 nm), with HMF, fructose and glucose. Based on ϵ values it can be calculated that yield of HMF under the assay conditions from fructose and glucose is 47.6% and 0.244%. With HMF, colour intensity reached a maximal value in 5 minutes, indicating that condensation of HMF formed

from hexoses is not the limiting phase in the overall reaction. The optical density values in o-cresol : 70% sulphuric acid reaction were linear with respect to fructose concentration in the range, 2.0-25 μ g of fructose with a ΔA_{500} value of 0.34 for 10 μ g.

The colour intensities produced by other sugars in o-cresol : 70% sulphuric acid reaction are compared with that of fructose in Table 1.

Table 1. Color intensities of sugars measured at 500 nm in o-cresol : 70% sulphuric acid reaction at 32°C after 15 minutes.

Sugar	Absorbance	Molar absorptivity	Relative color intensity
Fructose, 10 μ g	0.340	24500	100
Sucrose, 20 μ g	0.325	23400	95.5
Galactose, 200 μ g	0.050	180	0.74
Mannose, 200 μ g	0.145	520	2.12
Fucose, 200 μ g	0.093	310	1.26
Glucose, 200 μ g	0.037	130	0.54
Ribose, 200 μ g	0.322	970	3.96
Fructose, 10 μ g+ Glucose, 50 μ g	0.348	-	-
Fructose, 10 μ g+ Glucose, 400 μ g	0.420	-	-

Chromogen yield from sucrose is comparable to that of fructose on a molar basis, indicating that fructose portion of sucrose is responsible for the reaction. Among the hexoses, mannose was more chromogenic than galactose and glucose. Ribose produced appreciable color and it is only 25 times less sensitive than fructose. Addition of glucose to fructose caused small increase in absorbance and the values were in the expected range.

Total free carbohydrates and fructose, in serum samples were estimated using o-cresol : 75% sulphuric acid medium (see under methods) and the present method, respectively. The values are given in Table 2. Total carbohydrate level in normals, varied from 55.4 to 88.0 mg/dl. Fructose values (not corrected) ranged from 1.84 to 3.63 mg / dl.

Table 2. Serum free carbohydrates and fructose levels*

Group	Free carbohydrate	Fructose	Carbohydrate : Fructose Ratio
Normals (n=15)	68.8 $\pm$ 11.5	3.03 $\pm$ 0.485	23.0 $\pm$ 4.07
Diabetics (n=15)	257 $\pm$ 43.3	6.39 $\pm$ 1.39	40.9 $\pm$ 8.18

* Mean $\pm$ standard deviation.

Ratios of total carbohydrates to fructose were in the range, 16.5-30.3. Since glucose is the predominant carbohydrate in serum, a correction can be applied using the formula,

$$\text{Corrected fructose} = \frac{\text{Fructose} - (\text{Total carbohydrate} - \text{Fructose})}{185}$$

The corrected values ranged from 1.55 to 3.29 mg/dl. In diabetic samples, corrected and uncorrected fructose values were in the range, 2.91-6.81 and 4.18-8.65, respectively. Total carbohydrate ranged from 210 to 354 mg/dl. These values were slightly higher (average 7.10%) than the blood glucose values determined by the o-toluidine method, which ranged from 197 to 340 mg/dl. The ratios of total carbohydrate to fructose in diabetic samples varied from 28.1 to 57.2. Increase in both total carbohydrate and fructose were statistically significant ($p < 0.01$ and < 0.01 , respectively). However, increase in fructose level is much less compared to increase in total carbohydrate, which reflects mainly glucose.

DISCUSSION

The present studies indicate that the sensitivity of the colorimetric method using o-cresol and 70% sulphuric acid, has to be sacrificed to some extent, to ensure selectivity in terms of colour intensities of fructose and glucose. While at the suggested conditions, the selectivity ratio is 185 and the sensitivity as ϵ for fructose is 24500, increase in temperature to 45°C, increases to 34700, but curtails selectivity ratio to 42. Conversely, decrease in

acid level to 65% reduces ϵ to 13000 but enhances selectivity ratio to 295. The reported ratios of colour intensities for fructose and glucose are 90 by the anthrone : sulphuric acid method (5), 96 by the urea : perchloric acid method (4) and 173 by carbazole : sulphuric acid method (1). In the phenol : acetone : sulphuric acid method (3) the ratio is 4.5, whereas inclusion of boric acid in the medium increases the value to 160. However, in our method addition of boric acid reduces the ratio by enhancing color formation by glucose. It is not possible to compare the sensitivity of the o-cresol : sulphuric acid method with those reported by other methods due to differences in final volume and uncertainty regarding pathlengths of cuvettes used. Based on calculated ϵ values assuming a one cm pathlength the present method is more sensitive than the urea : perchloric acid and carbazole : sulphuric acid methods (1,3). It is comparable in sensitivity to anthrone : sulphuric acid method (5).

Normal serum fructose values estimated by the present method are comparable to the values reported by a specific enzymatic method (12). In diabetes, the levels are found to increase, which however is less significant than the increase in total free carbohydrate.

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