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Characterization and improvement of phenol-sulfuric acid microassay for glucose-based glycogen

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Abstract. – OBJECTIVES: Phenol-sulfuric acid reagent is used to measure the concentration of glyco-polymers and -conjugates. There are several uncertainties on glycogen measurement in the tissues. We aimed to improve phenol-sulfuric reagent for microassay of glucose based-glycogen in small tube or microplate.

MATERIALS AND METHODS: The condition of the reaction was optimized and scaled down for both small tube and microplate application.

RESULTS: The color intensity was found to be a function of all components of the assay mixture, that is, the amount of sugar and phenol together with the volume of total water and acid. The absorbance increased in the range of 4-10 mg of phenol and reached the plateau between 10-16 mg per 1 mL of acid. The color intensity was a linear function of total water volume (sugar-water- phenol). The sensitivity increased eight times as total water volume was changed from 50 up to 400 µL. The curve for acid volume peaked at about 1 mL. The optimal assay condition was determined to be 13 mg of phenol (200 µL 6.5%), 400-425 µL of total water volume (100 µL of sugar, 100 µL water) for 1 mL of acid. The initial spontaneous high temperature is essential the reaction to proceed and any handling gives inconsistent results and decreases the precision and sensitivity of the method. The values were scaled down by a factor of 0.5 for tube application and reading in cuvet or microplate and by 0.2 or 0.15 for microplate application.

CONCLUSIONS: The results indicated that phenol-sulfuric acid reagent could be scaled down to 1.0, 0.5 and 0.20, 0.15 mL of sulfuric acid for microassay of glucose based-glycogen.

Key Words:
Glucose, Glycogen, Phenol-sulfuric, Microassay, Microplate.

Introduction

Carbohydrates are quantified by chemical or enzymatic methods. The phenol-sulfuric acid reagent is widely used as a chemical method for the measurement of the sugars of polysaccharides, glycoproteins, proteoglycans and glycolipids[1]. The method is straightforward and sensitive for determining small quantity of sugars and their derivatives that have been separated by chromatography. It is also used to measure the total content of sugars in mixtures or biological samples[2]. Nevertheless, the reproducibility and hence the precision of the method depends on several parameters of the process.

In conventional method proposed by DuBois et al[1], to 1-2 mL of sugar in water, 1 mL of 5% phenol was added, followed by 5 mL of concentrated sulfuric acid. The mixture is shaken and allowed to progress by heat of reaction, after 30 min the absorbance read at 490 nm. Afterward, the method is adjusted for 3 and then 1 mL of sulfuric acid.

Several modifications have been described to increase the sensitivity and to reduce the variability of the assay toward different sugars in mixtures[3]. Rao and Pattabiraman[4] proposed that the heat is essential for dehydration of sugars into furfural but not for condensation with phenol. Hence, they cooled the mixture of acid and sugar-water after one minute and then added phenol to avoid sulfonation of phenol. Taylor[5] modified the volume of acid and showed that a temperature about 100°C is essential to achieve high sensitivity. The microplate scale proposed for antheron-, resorcinol- and phenol-sulfuric acid reagent[6-8]. Fox and Robyt[9] compared three micromethods to measure three sugars. In the current study, the condition was optimized for the assay of glucose-based glycogen and scaled down for small tube and microplate application.

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Materials and Methods

Materials

Perchloric acid (70%), concentrated sulfuric acid 85% (p = 1.834), ethanol, phenol and glucose were purchased from Merck Co (Darmstadt, Germany), glycogen from Sigma Co (Munich, Germany).

Rat Liver Sampling and Processing

The liver was isolated from male Sprague-Dawley rats, minced twice in 0.9% saline, dried on filter paper, weighted and ground quantitatively in 2 mL of 10% perchloric acid. The homogenate was centrifuged 10 minutes at 280 g, and the supernatant collected and extracted with 1.1 volumes of ethanol. The pellet was suspended in distilled water and analyzed for glycogen[10].

Optimized Glycogen or Glucose Assay

The condition for assay of glucose-based glycogen were optimized by combining 100 µL of glucose or glycogen (10 mg/dL), 100 µL of water, 200 µL of 6.5% phenol and 1 mL of 85% concentrated sulfuric acid ($H_2SO_{4(C)}$) in small (0.6×7 cm) tubes. A blank without glucose was set for each sample. The ratio of sugar-water; phenol-water; acid was 1; 1; 5. The ratio was scaled down to 0.5 mL of sulfuric acid for application in tube and reading in cuvet or microplate and also into 200 and 150 µL of acid for performing in microplate. For the microassay, an 8-well of 96-well polystyrene plate was used, and the absorbance was read with a microplate reader (Anthos, 2020, Salzburg, Austria) at 492 nm.

Statistical Analysis

The results are presented as the means ± SD of three inter-assays performed at least in three different preparations. The significant differences between samples and corresponding control were accessed by Student t-test. $p < 0.05$ was considered statistically significant.

Results

Effect of Phenol Concentration

The assay medium contained 10 µL of glucose (100 mg/dL), 200 µL of phenol-water and 1 mL of $H_2SO_{4(C)}$. The amount of phenol was changed in the assay medium from 4 up to 24 mg in constant total water volume of 210 µL. A blank without glucose was set for each sample. After 30 min the absorbance was read at 490 nm. Figure 1 shows that the absorbance is a function of phenol concentration. The color intensity was increased with the amount of phenol and reached the plateau at 10 mg of phenol and decreased over the succeeding 16 mg very slowly. The optimal amount of phenol was selected to be 13 mg.

Effect of Total Water Volume

The volumes of 0, 0-400 µL distilled water were added to 10 µL of glucose (100 mg/dL). Then 50 µL 26% phenol was added to the first tube and 100 µL 13% phenol to the other tubes and followed by adding 1 mL of $H_2SO_{4(C)}$. A blank without glucose was set for each sample. By this means, the total volume of aqueous medium (glucose-water-phenol) was changed from 50 up to 500 µL, but phenol amount was kept constant as 13 mg. Figure 2 shows that the absorbance is linear function of the total aqueous volume for the range 100 to 400 µL. The absorbance and hence sensitivity was increased about eight times with the increasing of water volume from 50 up to 400 µL, thereafter it peaked and then declined. The color of the assay medium changed from green-blue to green-orange at total water volume of 400 µL but the λ_{max} was not changed (results not shown).

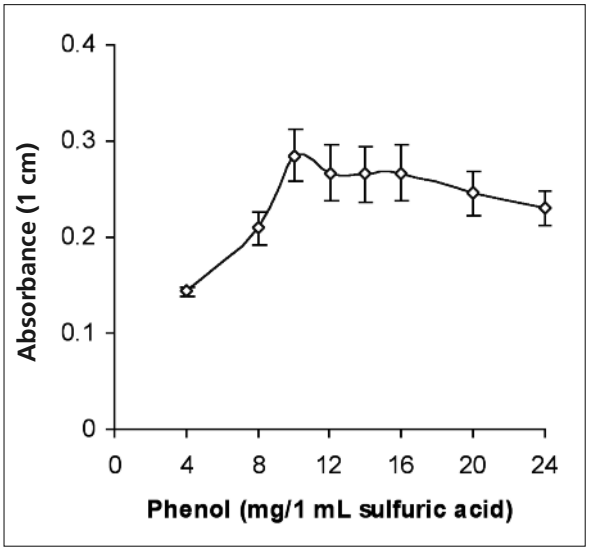

Figure 1. The effect of phenol amount. The assay medium contained 10 µg of glucose, different amounts of phenol and 1 mL of sulfuric acid. The total volume of aqueous medium (phenol-glucose-water) was kept constant as 210 µL, but phenol was varied from 4 up to 24 mg. A blank without glucose was set for each sample.

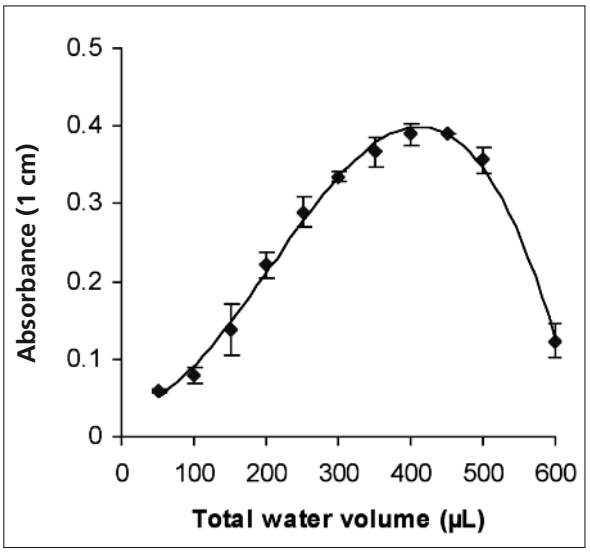

Figure 2. The effect of total water volume. The assay medium contained 10 µg of glucose, different volumes of water, 13 mg phenol and 1 mL of sulfuric acid. A blank without glucose was set for each sample.

Effect of Acid Volume

The effects of phenol amount and total water volume were studied in the presence of a constant volume of 1 mL acid. The effect of acid volume was also examined to provide a check on the previous results. To 100 µL of glucose (10 mg/dL) was added 100 µL water, 200 µL 6.5% phenol and 0.8-1.3 mL of $H_2SO_{4(C)}$. A blank without glucose was set for each sample. Figure 3 shows that the maximum absorbance is achieved at 1.0 mL of acid. From these data, 100 µL of sugar (10 mg/dL), 100 µL water, 200 µL 6.5% phenol and 1.0 mL of $H_2SO_{4(C)}$ was selected for the best sensitivity.

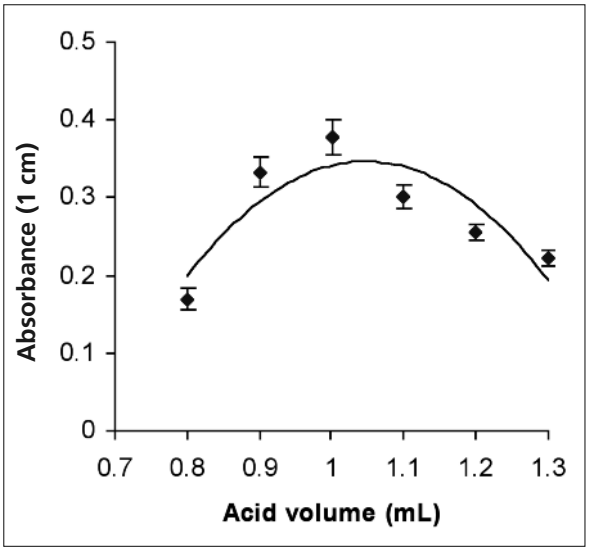

Figure 3. The effect of acid volume. The assay medium contained 100 µL of glucose (10 mg/dL), 100 µL water, 200 µL 6.5% phenol and different volumes of $H_2SO_{4(C)}$. A blank without glucose was set for each sample.

Sequence of Reagent, Temperature and Incubation Time

To enhance the reproducibility and sensitivity of the method, the time and temperature of the initial incubation were changed using optimized values (Table I). The conventional method optimized here is as follows. To 100 µL of sugar, 250 µL water, 50 µL 26% phenol was added 1 mL of $H_2SO_{4(C)}$, shaked to mix and allowed to stand for 30 min. Theses values were used with the following different procedures.

To control the high initial spontaneous temperature upon addition of acid to the aqueous medium, the reaction mixture was incubated immediately 5 min at 95°C and then allowed to stand for 25 min at room temperature. Masuko et al[8] did likewise but phenol was added as the last component. Alternatively, to control the initial temperature as the interesting experience of Taylor[5], acid was added to water, cooled and added to sugar and phenol. The reaction mixture was incubated 10 min at 95°C and then allowed it to stand for 20 min at room temperature.

In the Rao and Pattabiraman method[3,4], acid was added to sugar and water, the reaction was allowed to proceed by spontaneous heat and after 2 min the mixture was cooled to room temperature and phenol was added and allowed to stand for 30 min. The results show that the sensitivity was the most and the percent of CV was the least for the conventional method optimized by us (Table I).

Table I. The effect of sequence of reagent and temperature. 100 µL of sugar, 250 µL water, 50 µL 26% phenol and 1 mL of acid were used in all procedures.

	Absorbance (1 cm) X ± SD	Inter-assay % CV
Current study	0.366 ± 0.008	2.1
Incubated 5 min at 95°C	0.312 ± 0.018	5.7
Masuko et al	0.316 ± 0.018	5.5
Rao-Pattabiraman	0.217 ± 0.014	6.2
Taylor*	0.188 ± 0.028	14.6

*This is not optimized procedure of Taylor, but only is one of the methods described by him (reference 5).

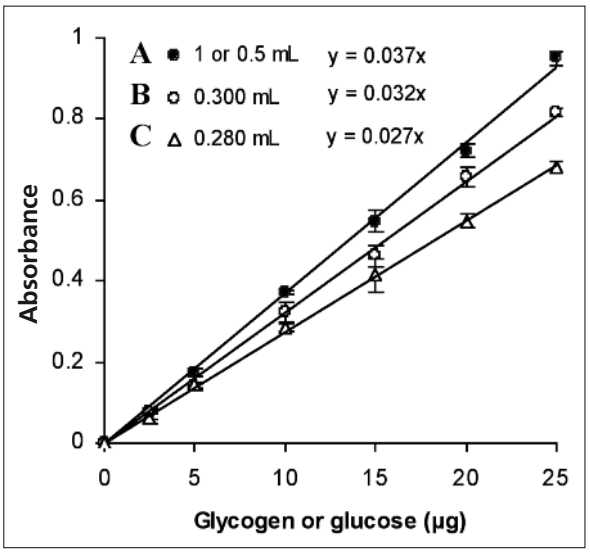

Figure 4. Standard concentration curve for glucose-based glycogen. ***A,*** The experiment performed in test tube using 0.5 (or 1) mL of sulfuric acid and read in 1 cm light path cuvet by LKB spectrophotometer. ***B,*** The final assay mixture (300 µL) of the previous experiment was transferred to microplate well to read the absorbance. ***C,*** The experiment was done totally in microplate in total volume of 280 µL.

Formatting the Reaction Condition for Microassay

The reaction conditions were scaled down to 0.5 mL of sulfuric acid for application in test tube and reading in cuvet or microplate and also to 200 and 150 µL for performing and reading in microplate. In a small test tube, was added 50 µL of glucose (10 mg/dL), 50 µL water, 100 µL 6.5% phenol and 0.5 mL of $H_2SO_{4(C)}$, shaken to mix and allowed to stand for 30 min. The absorbance was read in 1 cm light path cuvet, and the results were as the same as the experiment using 1 mL of $H_2SO_{4(C)}$ (Figure 4A). It may be difficult to transfer the assay mixture into cuvet due to its low volume, acidity and viscosity. Hence, 300 µL of the final reaction mixture was transferred into microplate well to read the absorbance by the reader. The standard curve was presented in Figure 4B.

The values were also scaled down by factors of 0.2 and 0.15 to run the experiment in microplate. A thin layer of cotton was put around the wells of the plate and moistened with distilled water to avoid deforming by initial librated heat. In each well was added 10 µL glucose (1 µg), 30 µL water, 40 µL of 6.5% phenol mixed and added 200 µL of acid with multichannel sampler. The mixture was shaken gently but completely. The wet cotton removed immediately from the plate. The plate allowed to stand at room temperature for 30 min. The standard curve is shown in Figure 4C. The curves are linear for a wide concentration range of glucose and glycogen. The correlation coefficients were more than 0.99 and intra-assay coefficient of variance (% CV) were less than %5 for all curves.

Discussion

The amount of phenol varies in assay medium from 8 up to 15 mg per 1 mL of sulfuric acid in different studies[1-5]. The sugars were separated by chromatography in the traditional method, and some phenol remained as solvent in the sample which was added to the reagent in the subsequent assay[1]. For this reason, Du Bois et al[1] selected the phenol concentration at the peak and beginning of plateau as 200 µL of 5% (equivalent of 10 mg) per 1 mL of acid. Masuko et al[8] to show the effect of phenol concentration added incorrectly different volumes of 5% phenol solution. As a consequence, not only the amount of phenol but also volume of total water was changed, for this reason they did not observe a plateau. Since it is preferred that the absorbance be independent of reagent concentration, the best amount of phenol was selected as 13 mg per 1 mL of sulfuric acid. This is equivalent of 200 µL of 6.5% phenol.

The results of the present study show for the first time that the color intensity is a strong function of the total volume of aqueous medium (sugar-water and phenol) (Figure 2). Although an appropriate blank must be set for each sample, the results could be compared if only the volume of total water is equal. The best condition for maximum sensitivity was deduced as 400-450 µL of total water volume and 12-14 mg of phenol per 1 mL of sulfuric acid. Water participates in the reaction; but it has a more critical role in heat production and control of temperature. The reproducibility of the results is also influenced by the rate of acid addition, the extent of vortex and the size of the tube). Long and severe vortex causes the assay mixture to rise higher in the tube, and the loss of the heat reduces the progression of color reaction.

Rao and Pattabiraman[3,4] proposed that the heat is essential for dehydration of sugars into furfural and its derivatives but not for condensa-

tion with phenol. Hence, they added acid to sugar-water and allowed the reaction to progress by spontaneous heat during one minute, then cooled the mixture and added phenol. Taylor[5] mixed acid and water to make 75% acid and cooled it. Then, was added to sugar and phenol and incubated at different temperatures. Although they obtained more agreeable values for equimolars of different sugars, the sensitivity for glucose is no longer was increased. We also changed the sequences of reagent addition, time and temperature of the initial incubation. The present data agree with the previous report indicate that handling of the initial temperature gives inconsistent results and decreases the precision of the method[5]. Table I shows that the sensitivity and precision was the best for the traditional procedure optimized here.

The conventional method proposed by Dubois et al[1] based on using of 5 mL of sulfuric acid; it was scaled down to 3 mL later. We optimized the reaction condition and scaled it to 1.0 and 0.5 mL to do in small tubes and also to 0.20 and 0.15 mL of acid to perform in microplate.

We recommend that, the experiment perform in small tube as 1 or 0.5 mL of acid and the absorbance read in microplate

Acknowledgements

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

References

1) Du Bois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956; 28: 350-356.
2) Saha SK, Brewer CF. Determination of the concentration of oligosaccharides, complex type carbohydrates, and glycoprotein using the phenol-sulfuric acid method. Carbohydr Res 1994; 254: 157-167.
3) Rao P, Pattabiraman TN. Reevaluation of the phenol-sulfuric acid reaction for the estimation of hexoses and pentoses. Anal Biochem 1989; 181: 18-22.
4) Rao P, Pattabiraman TN. Further studies on the mechanism of phenol-sulfuric acid reaction with furaldehyde derivatives. Anal Biochem 1990; 189: 178-181.
5) Taylor K. A modification of the phenol/sulfuric acid assay for total carbohydrates giving more comparable absorbance. Appl Biochem Biotechnol 1995; 53: 207-214.
6) Monsigny M, Petit C, Roche AC. Colorimetric determination of neutral sugars by a resorcinol sulfuric acid micromethod. Anal Biochem 1988; 175: 525-530.
7) Laurentin A, Edwards C. A microtiter modification of the anthrone-sulfuric acid colorimetric assay for glucose-based carbohydrates. Anal Biochem 2005; 315: 143-145.
8) Masuko T, Minami A, Iwasaki N, Majima T, Nishimura S, Lee YC. Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. Anal Biochem 2005; 339: 69-72.
9) Fox JD, Robyt JF. Miniaturization of three carbohydrate analyses using a microsample reader. Anal Biochem 1991; 195: 93-96.
10) Rasouli M, Mosavi-Mehr M, Tahmouri H. Liver denervation increases the levels of serum triglyceride and cholesterol via increases in the rate of VLDL secretion. Clin Res Hepatol Gastroenterol 2012; 36: 60-65.