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Original Research Article

Enhancing sensitivity of phenol-sulfuric acid assay using orthophosphoric acid as modifier

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ABSTRACT

Background: Phenol-sulfuric acid assay has been widely used for quantification of sugars in biological fluid and industry. The conventional method originally proposed by Dubois et al, was modified several times for enhancing sensitivity of assay such as substituting phenol with other chromogens, by optimizing assay conditions and by adding phenol after dehydration reaction. Both conventional and modified assays have utilized acid catalysis property of sulphuric acid but effect of adding phosphoric acid has not been studied before.
Methods: The present study was conducted in Department of Biochemistry, ANIIMS, Port Blair and IGIMS, Patna. The method being developed in our study consisted of adding orthophosphoric acid to the reaction mixture before addition of sulphuric acid. The method was optimized with different amounts of orthophosphoric acid. Statistical analysis was done using SPSS and Microsoft Excel software.
Results: The current study found an enhancing effect (on sensitivity) of orthophosphoric acid in optimal concentration of 5.16 mmoles. Comparison among standard curves of methods that were compared showed that the curve was steepest for current study and average absorbance was 0.199±0.017 for conventional, 0.253±0.011 for method by Rasouli et al, and 0.290±0.013 for current study. Pooled serum analysis exhibited absorbance of 0.157±0.015 in conventional method while in modified conventional method absorbance was 0.234±0.010 and highest absorbance was observed in current study at 0.281±0.012.
Conclusions: Our results suggest that orthophosphoric acid exerts a positively modifying effect on phenol sulphuric acid assay.

Keywords: Sulfuric acid, Orthophosphoric acid, Acid catalysis, Dehydration, Alcohol

INTRODUCTION

Quantification of sugars in biological fluid/sugar industry is accomplished widely by phenol-sulfuric acid method. This assay is commonly used for estimating total carbohydrates and carbohydrates linked to proteins as glycoproteins/glycosylated proteins in tissues or serum/plasma.[1] It has also been employed for measurement of glycated haemoglobin.[2] Presence of any monosaccaride or oligosaccharide having a C-2 hydroxyl group can be detected by phenol-sulfuric acid method.[3] The principle is based on dehydration of sugar in acidic medium forming hydroxymethylfurfural and its condensation with phenol to colored complex that can be measured at 490 nm.[1]

Available literature suggest that phenol-sulfuric acid originally proposed by Dubois et al, underwent various modifications in last six decades but the conventional method is in wide use.[1,4-11] The conventional method used a strong dehydrating agent as sulfuric acid and a chromogen as phenol. The conventional method was modified in several studies for enhancing sensitivity of assay; modifications were done either by substituting

phenol with other chromogens as resorcinol, antherone and 2,6-dimethylphenol or by altering assay conditions as modifying volume of acid/water and adding phenol after dehydrating sugar with acid.[5-11] Of all the modified methods, modification of conventional method by Rassouli et al, reported highest sensitivity and their study optimized the assay conditions such as water to acid ratio and phenol concentration.[10]

Dehydration of alcohol is acid catalyzed and commonly used mineral acids for dehydration are sulphuric acid and phosphoric acid. In conventional phenol sulphuric acid assay, dehydrating property of sulphuric acid is utilized but effect of adding other dehydrating agent i.e., phosphoric acid has not been studied before. The current study would investigate effect of adding orthophosphoric acid in phenol sulphuric acid assay on sensitivity of assay, if any, and compare it with conventional method developed by Dubois et al and method optimized by Rassouli et al.

METHODS

The present study was conducted in Andaman & Nicobar Islands Institute of Medical Sciences, Port Blair and Department of Biochemistry, Indira Gandhi Institute of Medical Sciences, Patna. All reagents used in present study were analytical grade. The method being developed in our study consisted of adding orthophosphoric acid to the reaction mixture before addition of sulphuric acid. The method was optimized with different amounts of orthophosphoric acid. The optimized protocol is described here for a 2.8 ml assay; to a 200 µl of 6 mg% standard glucose (12 µg glucose) add 400 µl of 7% phenol solution, 100 µl water and 350 µl of 85% orthophosphoric acid (5.16 mmoles), mix well and then pour 1750 µl of 85% sulphuric acid by the side of the tube and thereafter read absorbance at 490 nm after 20 minutes. The optimized method in current study was compared with conventional method and a modified conventional method, both of which were scaled to 2.8 ml total assay volume.[1,3,10] Briefly, 215 µl 7% phenol, 385 µl water, 200 µl standard (12 µg glucose) and 2 ml sulphuric acid for conventional and 400 µl 7% phenol, 200 µl water, 200 µl standard (12 µg glucose) and 2 ml sulphuric acid for modified conventional method. Serum was pooled from from ten serum samples with normal routinue biochemistry profile and analyzed on same day. Statistical analysis was done using SPSS and Microsoft Excel software. Intra-assay and inter-assay coefficient of variation were 3.9% and 4.1% respectively.

RESULTS

The method optimized with orthophosphoric acid showed a peak absorbance at 5.16 mmoles (equivalent to 350 µl) of orthophosphoric acid. All reaction mixtures were of identical assay volume of 2.8 ml. To each reaction mixture, a 28 mg phenol (400 µl of 7% phenol) and 12 µg of standard glucose (200 µl of 6 mg% glucose solution) was added. Different volumes of orthophosphoric acid were added to each tube in gradients from 0.37 mmoles to 11.8 mmoles. Consequently volumes of water and sulphuric acid were adjusted in each reaction mix to maintain a water:acid ratio of 1:2.5. The result suggest a enhancing effect (on sensitivity) of orthophosphoric acid in optimal concentration of 5.16 mmoles while the effect waned at suboptimal concentrations. Duplicates of blank without standard glucose was included for each reaction mixture (Figure 1).

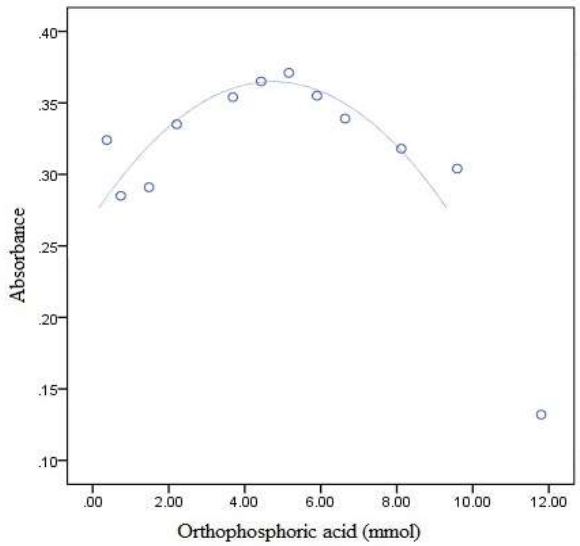

Figure 1: Optimization of phenol-sulfuric acid with different amounts of orthophosphoric acid.

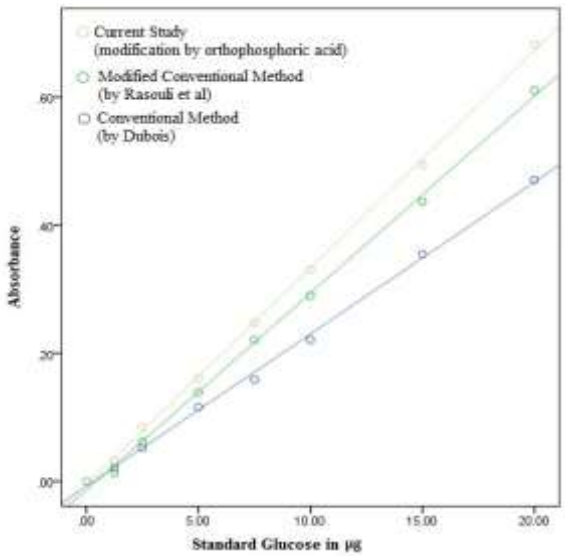

Figure 2: Comparison among standard curves of conventional, modified conventional and method modified by current study.

Comparison among standard curves of methods that were compared showed that curve was steepest for current study and average absorbance was 0.199±0.017 for

conventional, 0.253±0.011 for method by Rasouli et al, 0.290±0.012 for current study (Figure 2).

Correlation coefficient among all standard curves were near perfect (>0.99). Pooled serum analysis exhibited absorbance of 0.157±0.015 in conventional method while in modified conventional method absorbance was 0.234±0.010 and highest absorbance was observed in current study at 0.281±0.013.

DISCUSSION

The current study has examined role of orthophosphoric acid in modifying phenol sulphuric acid for the first time. Optimization process in our study has shown that addition of orthophosphoric acid in optimal concentration enhances the sensitivity of assay and optimal concentration for 2.8 ml assay was found to be 5.16 mmoles or 350 µl of 85% orthophosphoric acid (absorbance 0.371±0.013 for 12 µg glucose added) (Figure 1). The current study introduced orthophosphoric acid in phenol sulphuric acid assay without interfering the key tenets of reaction i.e., water to acid ratio of 1:2.5 and optimal phenol concentration of 10 mg/ml assay. It was also observed that absorbance was slightly decreased (0.362±0.014 for 12 µg glucose) when phenol was adjusted for actual water and sulphuric acid volumes rather than total assay volume of 2.8 ml i.e., actual water and sulphuric acid left in reaction mixture was 2450 µl after substracting 350 µl of orthophosphoric acid, consequently phenol used was 24.5 mg rather than 28 mg. It can be deduced from these observations that sensitivity of assay is dependent on water to acid ratio rather than total assay volume while for phenol, it is dependent on total assay volume.

Comparison of method developed in current study with conventional method and modified conventional method reveal sensitivity of assay to be highest in our study for standards ranging from 1.25 µg to 20 µg glucose (Figure 2). The absorbance in current study had 49.77% higher absorbance than conventional and 14.14% higher absorbance than modified conventional method. The enhancing effect also resonated when the assay developed in current study was applied on biological specimen i.e., pooled serum.

Sugars undergo dehydration when treated with conc. sulphuric acid to form furfural derivatives which on condensation with phenol forms a orange-yellow color.[1] Rao et al reported that in conventional phenol-sulfuric acid assay, phenol underwent sulfonation in situ that decreased the color intensity of hydroxymethyl furfural.[4,11] They also reported that though heating was a pre-requisite for dehydration and consequent formation of furfural but condensation of phenol with furfural was independent of heat. Thus they added sulphuric acid directly in sugar solution, the reaction mixture was cooled and then phenol was added. Rao et al proposed that this maneuver curtailed sulfonation of phenol.

Sulphuric acid acts as a good oxidizing agent during dehydration thus charring the reacting carbohydrate while phosphoric acid dehydrates but does not char alcoholic groups.[12] It is plausible that positively modifying action of orthophosphoric acid could be attributed to its properties on linear alcohols and aryl alcohols. Firstly, as dehydrating agent it could augment dehydration of sugar alcohols by sulphuric acid. Secondly, Sulphuric acid can sulfonate the phenol while phosphoric acid stabilizes phenol and it is plausible that part of this stabilization occurs by desulfonation because sulfonation is a reversible process in presence of water and phosphoric acid has traditionally been used in industry for dehydration of cyclohexanol to cyclohexene.[13-15] Thirdly, addition of phosphoric acid in our study has resulted in contraction in volumes of sulphuric acid and hence slightly lesser propensity to sulfonate phenol. It is beyond the scope of current study to formulate reaction mechanism while it could be generally stated based on our results that phosphoric acid positively modifies phenol sulphuric acid assay.

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