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Interference of Mannose and Galactose in Glucose Assay by the Glucose Oxidase/Peroxidase Method

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Objectives: Although the glucose oxidase (GOx) enzyme is produced by a variety of different organisms. The most commonly enzyme originates from the fungus *Aspergillus Niger* is widely implicated in glucose estimation. *A. niger* GOx enzyme should be highly sensitive and selective for glucose. However, GOx extracted from *A. Niger* may contain other enzyme impurities. Potential interfering agents as glucose epimers can impact the accuracy of results obtained by glucose oxidase- Peroxidase method.
Methods: Glucose epimers as galactose and mannose were *in vitro* screened for interference with glucose estimation by glucose Oxidase-Peroxidase method (GOx-POD) at concentrations similar

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to physiological concentrations of glucose. Furthermore, different concentrations of mannose and galactose calibration experiments were estimated by the enzymatic method implicated for glucose determination to understand the potential source and mechanism of interference from glucose epimers.

Results: Epimers of glucose as mannose and galactose can interfere with the glucose determination using the glucose oxidase/peroxidase (EC 1.1.3.4/1.11.1.7) (GOx-POD) method utilizing reduced o-Dianisidine as the oxygen acceptor chromogen. There was a linear relationship between the concentrations of mannose and glucose readings. Each 0.11 µmol/l of mannose in samples leads to an apparent increase of about 0.11 µmol/l of glucose. This interference in the GOx-POD method for glucose assay can occur in the in vitro experimental samples and cause over-estimation of the glucose. While the implicated enzymatic method glucose oxidase/peroxidase can detect only higher concentrations of galactose up to 0.275 µmol/l.

Conclusion: Under the conditions of in vitro measurement of glucose, using GOx-POD method, different concentrations of mannose (low and high) and while higher concentrations of galactose have a measurable interference. Mannose exhibited the highest interference during screening. Enzyme kinetic analysis conducted with galactose and mannose supported the notion that, the reactivity of GOx enzyme toward epimers of glucose and the presence of enzymatic impurities (such as galactose or mannose oxidase) are two potential sources for sugar interference with GOx. It is suggested that careful attention should be paid to the wide applicability of the GOx-POD method for glucose assay.

Keywords: Glucose oxidase; peroxidase; mannose; galactose.

1. INTRODUCTION

One of the clinically important biomarker in maintaining normal physiological activities of living systems is the glucose as it plays an important role [1]. Elevation of blood glucose level or any variation in its blood concentration may results in various metabolic disorders especially diabetes which is one of the most worldwide public health problem [2,3]. The diabetic complications include higher risk of retinopathy and blindness, cardiovascular disease, nephropathy leading to kidney failure. Therefore, measurement of blood glucose level is necessary in clinical diagnosis of diabetes mellitus. Hence, monitoring blood glucose level require rapid, selective and precise method [4,5]. One of the most common tests used in clinical practice is the enzymatic determination of blood glucose level because of its high sensitivity, specificity, and simplicity compared to the other chemical methods [6]. Moreover, several instrumental methods for the determination of glucose in serum samples have been reported [7–17]. However, several co-substrates are being used in the enzymatic determination of glucose [18–24].

Each method has some unique advantages, including using of less than 20 µl serum sample whereas long incubation period (more than 10 min) coupled with multiple steps involved in the procedure are the major disadvantages. Some of the methods have sophisticated techniques [7–16] requiring skilled operators to handle instruments, which are relatively expensive and requiring multiple steps for preparation of sensors and also need immobilization of enzymes.

The chemical methods [23,24] although rapid, suffer from serious interference by lactose, galactose, and glutathione. Recently, electrochemical non-enzymatic glucose sensors [25] have received attention because of their stability and simplicity as compared to enzyme-based sensors, but they also suffer from low sensitivity and selectivity [13].

Glucose analogues are commonly introduced to the human body, orally through diet. Sucralose, mannitol, aspartame, and stevia are glucose free substitutes. They are the major diet for people with diabetes, to help with glycemic control. Enzymatic interferences of non-glucose sugars with blood glucose meters that use the glucose dehydrogenase enzyme have been reported, in some cases leading to serious or fatal outcomes [26-27]. Therefore, it is important to identify the selectivity and sensitivity of glucose oxidase/Peroxidase method for glucose estimation and to determine the interference another monosaccharides. However, most of the methods were not investigated the interference of another monosaccharide with structural similarity as mannose, galactose and fructose

which are most common interfering compounds with glucose estimation by the enzymatic method glucose oxidase- Peroxidase (GOx- POD). Therefore, it is important to understand the susceptibility of GOx based method to similar interferences with glucose epimers.

The goal of this study is to evaluate the effect of glucose epimers (mannose and galactose) interference with GOx-POD-based method for glucose estimation. Commercial kit for glucose estimation were used to conduct this study, and measuring the percentage of interference of another monosaccharide as mannose and galactose by adding known amount to aliquot and measure the percentage of interference.

2. MATERIALS AND METHODS

2.1 Chemicals

Glucose, mannose and galactose powder was supplied by Sigma Chemical Company. For the stability study, double distilled water with pH ranging from 4.0 and 5.0 will be used for buffering.

The following reagents were prepared; glucose oxidase/peroxidase reagent was supplied from Sigma –Aldrich prepared by dissolving the content of capsule in an amber bottle with 39.2 ml of deionized water. While o-dianisidine 5 mg were dissolved in 1 ml of deionized water. The assay reagent mixture was prepared by adding 0.8 ml of o-dianisidine reagent with 39.2 ml of glucose oxidase/peroxidase reagent.

2.2 Reagents

1. Glucose Oxidase/Peroxidase Reagent (Catalog Number G3660)
   Each capsule contains 500 units of glucose oxidase (Aspergillus niger), 100 purpurogallin units of peroxidase (horseradish), and buffer salts. Empty the capsule contents into an amber bottle. Dissolve those contents in 39.2 mL of deionized water.
2. o-Dianisidine Reagent (Catalog Number D2679)
   The pre-weighed vial contains 5 mg of o-dianisidine dihydrochloride. Reconstitute the contents of the o-dianisidine vial with 1.0 mL of deionized water.
3. Assay Reagent
   Add 0.8 mL of the o-Dianisidine Reagent to the amber bottle containing the 39.2 mL of Glucose Oxidase/Peroxidase Reagent. Invert the bottle several times to mix.
4. Glucose Standard Solution (Catalog Number G3285) D-Glucose, 1.0 mg/mL in 0.1% benzoic acid.

2.3 Quantification of Glucose, Mannose and Galactose by Glucose Oxidase Kit

The Glucose Assay Kit provides a simple and direct procedure for measuring glucose in a variety of biological samples. Glucose is determined by a coupled enzyme assay, in which GOx oxidizes D-glucose resulting in the production of gluconic acid and hydrogen peroxide (H_2O_2) that reacts with o-dianisidine in the presence of peroxidase generating Oxidized o-dianisidine, which reacts with sulfuric acid to form more stable colored product proportional to the glucose concentration. One unit of GOx is defined as the amount of enzyme that generates 1.0 µmol of H_2O_2 per minute at 37 °C.

A simple spectrophotometric method for determination of all monosaccharides were applied based on the method presented by Nagaraja et al. [28] with minor modifications. A spectrophotometric method for mannose and galactose quantification based on generation of H_2O_2 in situ by oxidation reaction between glucose oxidase and mannose, galactose, which in the presence of peroxidase reacts with o-dianisidine in the presence of peroxidase generating oxidized o-dianisidine, which reacts with sulfuric acid to form stable colored product measured at 540nm.

The intensity of the color was proportional with all monosaccharides concentrations. To test interference of monosaccharide, mannose and galactose, known amount of different concentrations of monosaccharides added to 1 ml of the reaction mixture containing GOx-POD in 100 mmol/l generating hydrogen peroxide, which reacts with o-dianisidine in the presence of peroxidase to form colored product. The reaction mixture incubated for 10 min at 37 °C.

Preparation of Benedict solution: One liter of Benedict's reagent can be prepared by mixing 17.3 grams of copper sulfate pentahydrate ($CuSO_4.5H_2O$), 100 grams of sodium carbonate (Na_2CO_3), and 173 grams of sodium citrate in distilled water (required quantity). Different concentration of glucose, mannose and

galactose added to 1 ml of benedict solution in boiling water bath for 5 min. Benedict's test used to test for detection of presence of reducing sugar. It identifies reducing sugars, which have free ketone or aldehyde as functional groups. Usually it implicated for detection of glucose in urine. When reducing sugars are mixed with Benedicts reagent and heated, a reduction reaction occurs which causes the Benedicts reagent to change color. The color varies from green to dark red (brick) or rusty-brown, depending on the amount of and type of sugar.

3. RESULTS

The present results investigated the effect of various concentrations of mannose and galactose on glucose determination by the GOx-POD method in the in vitro experiments. The stock standard solution glucose concentration was prepared as 1mg/ml and different glucose concentrations were measured as (10, 20, 30, 40 and 50 µg of glucose concentrations: 55-275 nmol/l) as shown in Fig. 1. Under the same condition estimation of parallel concentrations of mannose, using same method indicated that GOx-POD could detect mannose at the same concentrations of glucose Fig. 2. The intensity of color produced by different concentration of mannose were 30% lower than that color observed with the same concentrations of glucose. Mannose was not expected to be detected enzymatically by glucose oxidase as it is consider to be highly selective for glucose. Mannose can interfere with glucose estimation although the sensitivity of method was 30% lower in mannose detection. On the other hand, galactose did not exhibit an interfering effect in lower concentrations during screening. Implicated enzymatic method GOx-POD can detect only higher concentrations of galactose (200-800 µg) Fig. 3. The simultaneous presence of mannose evidently interfered with the GOx-POD method. There was an essentially linear relationship between the concentrations of mannose and increasing glucose readings. Addition of equal concentrations of mannose and glucose leads to duplication of the absorbance Fig. 4. These interferences indicated that glucose oxidase implicated is not selective for glucose and can detect mannose. Our results indicate that mannose interfere with glucose estimation although the sensitivity of method was 30% lower in mannose detection. However, galactose addition to different concentrations of glucose cannot affect the absorbance.

To investigate the reducing power of different monosaccharide used, addition of different concentrations of glucose, mannose and galactose to 1 ml of Benedict solution and the intensity of the color produced were reported. Same concentrations of mannose and galactose have same potency for reducing Benedict solutions, while only higher concentration of glucose have the reducing power to change color Table 1.

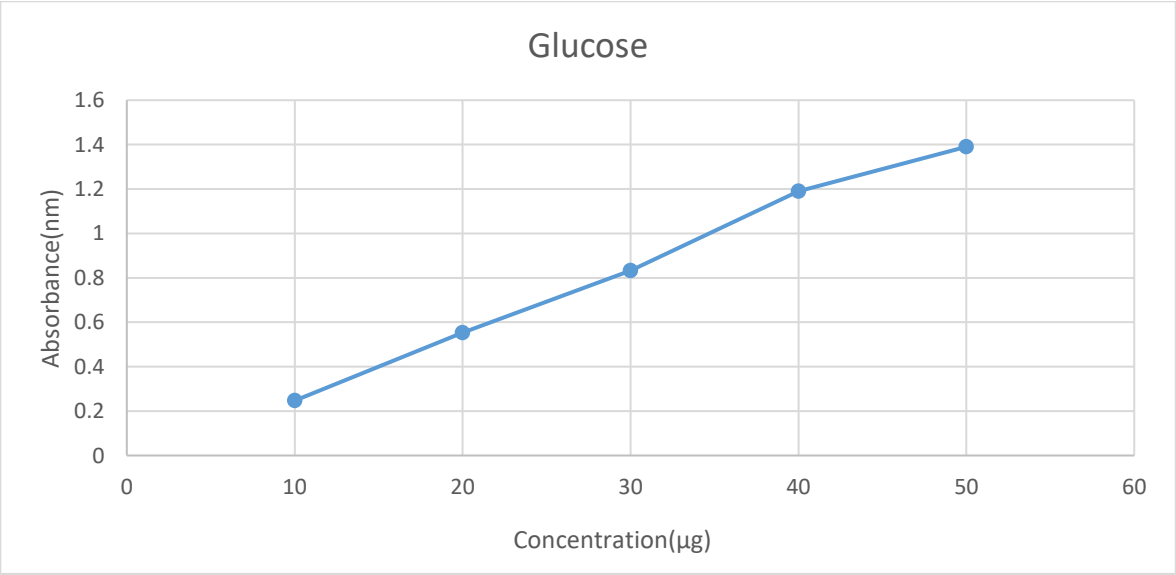

Fig. 1. Determination of different concentrations of glucose by GOx-POD method. GOx can detect low concentrations of glucose 10, 20 30, 40 and 50 µg

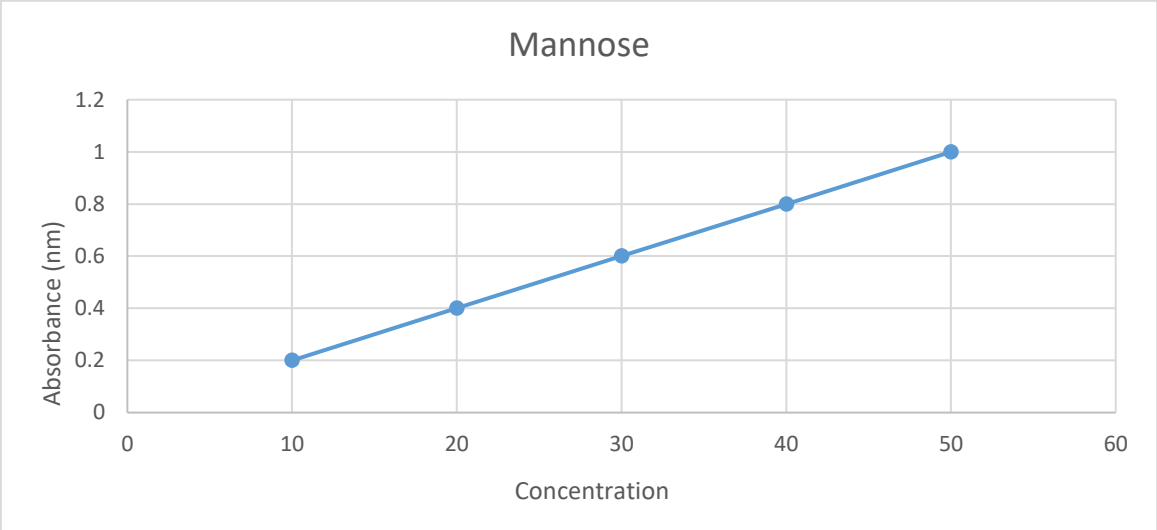

Fig. 2. Determination of different concentrations of Mannose by GOx-POD method. GOx can detect low and high mannose concentrations

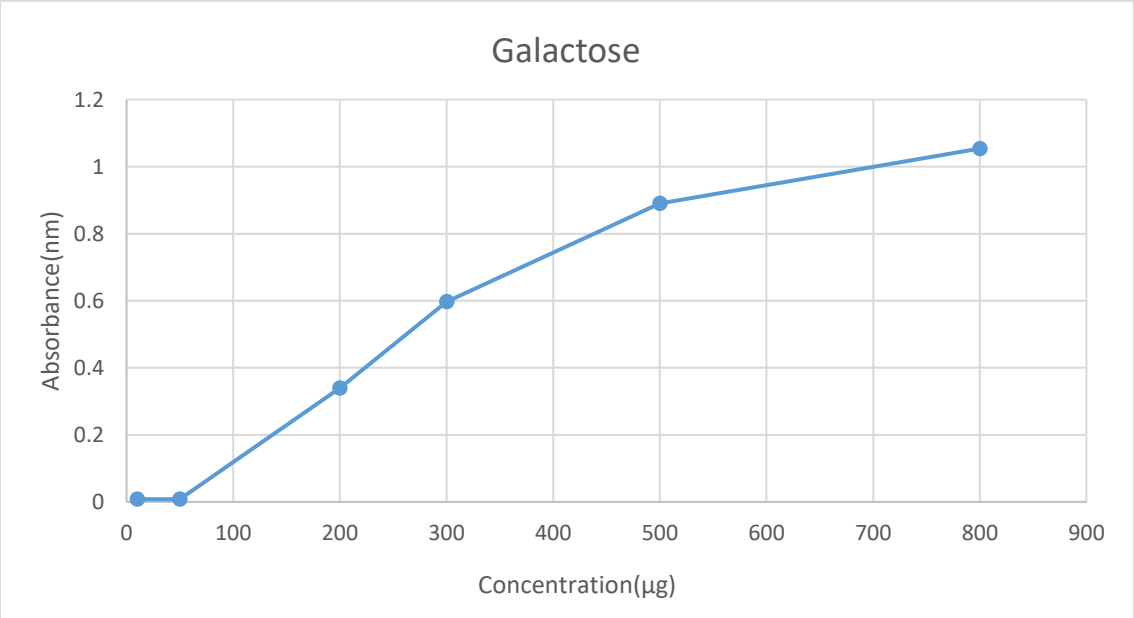

Fig. 3. Determination of different concentrations of Galactose by GOx-GOD method. GOx can detect higher concentrations of galactose above 100 µg

Table 1. Qualitative detection of different monosaccharides by Benedict reagent

Concentrations	Glucose	Mannose	Galactose
10 µg	-ve	-ve	-ve
20 µg	-ve	-ve	-ve
40 µg	-ve	+ve	+ve
60 µg	-ve	++ve	++ve
80 µg	-ve	+++ve	+++ve
100 µg	-ve	+++ve	+++ve
200 µg	-ve	+++ve	+++ve
400 µg	++ve	+++ve	+++ve

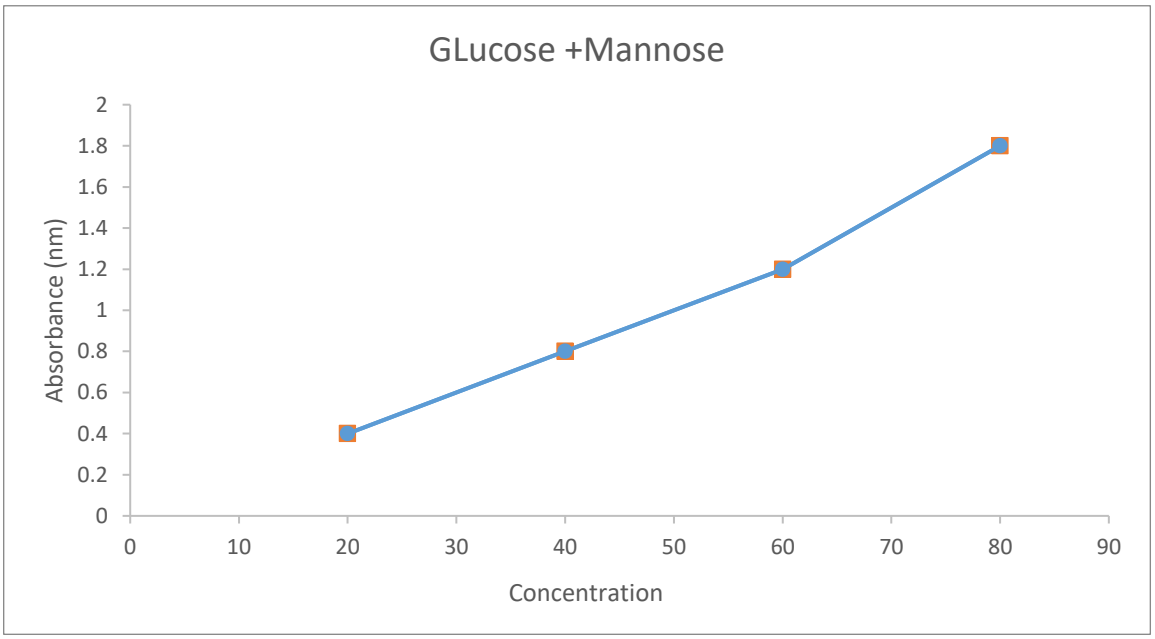

Fig. 4. Determination of mixed equal concentrations of mannose and glucose

4. DISCUSSION

Mannose is the monosaccharide of glycoproteins and glycolipids constituent. In humans, the mean plasma concentrations of mannose were shown previously to be approximately 50 [29], 20 [30] and 40 µmol/l [31]. Mannose plasma concentration estimated in diabetic patients have to be higher than participants with normal glucose tolerance are [31]. Youshimura et al. [32], showed that part of the plasma mannose is supplied by glycogenolysis as glycogen → glucose 1-phosphate → fructose 6-phosphate → mannose 6-phosphate → mannose.

The present study strongly suggested that low and high concentrations of mannose while higher concentrations of galactose interfere in the enzymatic method involved in glucose determination. They oxidized as glucose-by-glucose oxidase liberating hydrogen peroxide in preference to the chromogen. Although it was reported different organisms produce the glucose oxidase (GOx) enzyme especially fungus *Aspergillus niger which* is highly selective for glucose and exhibits limited reactivity toward other sugars [33-35]. However, GOx extracted from *A. niger* may contain other enzyme impurities [35]. Contamination of GOx with other enzymes may be consider the potential contributing factor to this interference. Galactose oxidase (GalOx) impurities in the GOx enzyme used in the glucose estimation may explain interference of higher concentrations of galactose. The oxidation of D-galactose by GalOx in the presence of O_2 results in production of D-galacto-hexodialdose and hydrogen peroxide (H_2O_2). Since this reaction produces additional H_2O_2, which could be then further, oxidize chromogen. Therefore, the possible presence of GalOx creates the potential for an increase in interfering signal with increasing galactose concentration. GOx stocks acquired from commercial sources are largely pure, but vendors do report lot-specific contamination by enzymes such as α-amylase, saccharase, maltase, glycogenase, invertase, protease, and galactose oxidase [35].

Moreover, we could propose that GOx can directly oxidize mannose, as there is no selective mannose oxidase reported before. Youshimura et al. [32], showed the *in vitro* interferences of seven non-glucose sugars, seven sugar alcohols, and three artificial sweeteners with amperometric GOx-based continuous glucose monitoring sensors. They reported that there were interferences of <20%. Our results can confirm the previous study demonstrated that very high, non-physiological concentrations of galactose, showed the highest levels of interference. While at physiologically relevant concentrations (0.1-100 µg), the galactose interference was largely negligible.

Previously published results indicated that interference appeared to diminish as glucose

concentration increased from hypoglycemic- to euglycemic to hyperglycemic-relevant concentrations, suggesting that interferences may have proportionately greater impact at lower rather than higher glucose levels. The potential for greater interferences at the low glucose concentrations may be attributed to either GOx reactivity toward galactose, and/or to the presence of GalOx impurities within the GOx enzyme layer. GOx reactivity and purity should be taken into consideration when designing GOx-based method for glucose detection. Additional studies are highly warranted to quantify the impact of interferences on glucose estimation.

5. CONCLUSION

These data based on *in vitro* testing protocols. Since *in vitro* data do not always correlate with *in vivo* activity, *in vivo* studies would ultimately determine the clinical significance of any interferences.

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CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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