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The utilization of bathocuproinedisulfonic acid as a reagent for determining D-glucose and D-galactose levels in glycoconjugates

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Abstract

A sensitive, rapid, and reliable method for measuring D-glucose and D-galactose levels in glycoconjugates has been developed. In this method, the NAD(P)H produced from the enzymatic oxidation of the monosaccharides is reacted with a $CuSO_4$-bathocuproinedisulfonic acid reagent (Cu-BCS) to produce a color complex absorbing maximally at 486 nm. With galactose dehydrogenase and glucose dehydrogenase serving as the model enzymes, graphs of absorbance versus varying D-glucose or D-galactose concentrations yielded a linear plot from 2.5 to 250 nmol of sugar. Using this procedure, sugar released by acid hydrolysis from lactose, porcine submaxillary mucin and raffinose was quantified. When *p*-nitrophenyl-α-D-glucopyranoside and *p*-nitrophenyl-β-D-galactopyranoside were acid hydrolyzed and assayed with the Cu-BCS reagent, the amount of sugar released from each of the *p*-nitrophenyl compounds was found to be equal to the levels of *p*-nitrophenol in solution. This method is easy to use and with minor modifications can be employed for the quantification of D-glucose and D-galactose in other glycoconjugates.

Keywords: Nicotinamide adenine dinucleotide; Nicotinamide adenine dinucleotide phosphate; Glucose dehydrogenase; Galactose dehydrogenase; Raffinose; Lactose; Porcine submaxillary mucin; *p*-Nitrophenyl-α-D-glucopyranoside; *p*-Nitrophenyl-β-D-galactopyranoside; Bathocuproinedisulfonic acid

1. Introduction

The determination of D-glucose and D-galactose levels in glycoconjugates has been achieved by several liquid chromatographic and enzymic methods [1–7]. Of the various chromatographic methods that have been used for the sensitive determination of sugars in biological samples, HPLC with refractive index detection has received considerable attention. For example, using this approach, Chávez-Servín et al. [6] detected as low as 0.13 mg/mL of D-glucose and 0.06 mg/mL of D-galactose in milk based formula, with the quantification limits of the assay being 0.24 and 0.20 mg/mL for D-glucose and D-galactose, respectively. Nevertheless, one drawback to most liquid chromatographic methods is that, relative to enzymic assays, they are typically more labor intensive and require the separation of carbohydrates prior to their analysis. One common enzymic method for the determination of D-glucose and D-galactose has relied on the oxidation of the sugars by specific dehydrogenase enzymes, yielding NAD(P)H which can be monitored by UV spectrophotometric, fluorometric and colorimetric assays [8–13].

The direct measurement of NAD(P)H by spectrophotometric and fluorometric techniques has allowed for several sensitive assays, with each method yielding detection limits as low as 1 and 0.1 nmol for NAD(P)H, respectively [2,14,15]. Although versatile and sensitive, the direct detection of NAD(P)H does not provide for a visually detectable color change which can prove beneficial, in instances where qualitative analysis of glycoconjugates is sought. Additionally, the use of fluorometry as an analytical tool has been shown to be limited by a significant dependence upon environmental conditions such as temperature, pH, ionic strength and quenching due to impurities [1,12,16].

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While the colorimetric measurement of NAD(P)H has been achieved by various tetrazolium salts, one drawback to these methods is their typical requirement for diaphorase, an enzyme used for the transfer of electrons from NAD(P)H to the tetrazolium salts [1,11].

We describe here a novel colorimetric method, which avoids the shortcomings of the UV and fluorometric assays and which allows for the rapid and accurate measurement of NAD(P)H in the absence of diaphorase. In this method, the NAD(P)H formed from the specific oxidation of the sugars is reacted with Cu^{2+} in the presence of bathocuproinedisulfonic acid (BCS) to form a color complex absorbing maximally at 486 nm. With galactose dehydrogenase [EC 1.1.1.48] and glucose dehydrogenase [EC 1.1.1.47] serving as the model enzymes (respectively, requiring NAD^+ and $NADP^+$ as a cofactor), this assay was used to quantify D-galactose and D-glucose in several natural and synthetic compounds. Reactions 1–3 below summarize the general principle of the assay with D-galactose as the test sugar.

$$\text{D-galactose} + NAD^+ \xrightarrow{\text{galactose dehydrogenase}} \text{D-galactonate} + NADH + H^+ \quad (1)$$

$$NADH + Cu^{2+} \rightarrow NAD^+ + Cu^+ \quad (2)$$

$$Cu^+ + 2BCS \rightarrow Cu^+\text{-}BCS_2 \text{ complex} \quad (3)$$

In this paper we describe the application of the Cu-BCS method for the quantification of D-glucose and D-galactose in lactose, raffinose, porcine submaxillary mucin (PSM) and *p*-nitrophenyl glycoconjugates.

2. Materials and methods

2.1. Chemicals and supplies

Unless otherwise indicated, all materials employed were of analytical grade and were purchased from Sigma–Aldrich Co. (St. Louis, MO).

2.2. Copper-BCS reagent

All solutions were prepared fresh as follows: 76 mg of $Cu(SO_4)$ was dissolved in 5.0 mL distilled-deionized water. To this, 454.4 mg of BCS was added, and the solution was brought to a final volume of 100 mL using 0.2 M sodium acetate buffer, pH 4.7. The concentrations of $Cu(SO_4)$ (4.76 mM) and BCS (8.05 mM) were carefully selected to ensure that no copper or BCS precipitated in the color reagent and optimal color was produced when the Cu-BCS reagent was used.

2.3. Colorimetric measurement of free D-galactose and D-glucose by galactose and glucose dehydrogenase, respectively, and the Cu-BCS reagent

A standard reaction mixture contained 0.25 μmol D-galactose, 0.86 μmol NAD^+, 0.1 U galactose dehydrogenase from *Pseudomonas fluorescens* and 10 mM Tris buffer, pH 8.5, in a total volume of 1.5 mL. This solution was incubated at 25 °C for 90 min, at which time all D-galactose in the incubation mixture was oxidized. The NADH generated by galactose dehydrogenase was then reacted with 1.5 mL of the Cu-BCS reagent and the absorbance of the resulting mixture was measured at 486 nm using a Hitachi U-2810 spectrophotometer. Measurement of free D-glucose by glucose dehydrogenase was carried out in an identical manner to the D-galactose assay except the reaction was performed at 37 °C for 120 min with D-glucose and $NADP^+$ serving as substrates. Unless otherwise indicated, a unit (U) of galactose dehydrogenase or glucose dehydrogenase activity is defined as the amount of enzyme that oxidizes 1 μmol of sugar per min at pH 8.5 at 25 and 37 °C, respectively.

2.4. Colorimetric measurement of D-galactose/D-glucose in lactose, raffinose, and porcine submaxillary mucin by galactose/glucose dehydrogenase and the Cu-BCS reagent

Solutions of lactose (various concentrations), raffinose (0.74 mg/mL) and PSM (2.5 mg/mL) were acid hydrolyzed in 0.2 N HCl for lactose and in 0.5 N HCl for raffinose and PSM at 100 °C. The hydrolysis time for lactose was 120 min and for raffinose and PSM, hydrolysis was 75 min. Aliquots (0.2 mL) were removed from the lactose mixtures following their complete hydrolysis and from the raffinose and PSM mixtures over time. Samples were then neutralized using 1 N NaOH (~75 uL) and assayed with galactose and/or glucose dehydrogenase using the Cu-BCS reagent as previously described.

2.5. Monitoring the acid hydrolysis of PNP-α-D-glucopyranoside and measuring released D-glucose

PNP-α-D-glucopyranoside (0.32 mg/mL) was hydrolyzed in 0.2 N HCl at 100 °C for 60 min. At different intervals, 0.1 mL aliquots were withdrawn and each was brought up to 1.5 mL with 50 mM Tris buffer, pH 8.5 containing 0.86 μmol of $NADP^+$ and 0.1 U of glucose dehydrogenase. Mixtures were incubated for 120 min at 37 °C at which time 1.5 mL of the Cu-BCS reagent was added. In a separate experiment conducted under equivalent incubation conditions, the 0.1 mL aliquots were immediately mixed with 2.9 mL of 0.5 M glycine buffer, pH 10, and assayed for *p*-nitrophenol at 400 nm.

2.6. Identification of D-galactose/D-glucose as the product(s) released from lactose, raffinose, and porcine submaxillary mucin by descending paper chromatography

Solutions of lactose (51.9 mg/mL), raffinose (73.3 mg/mL), and PSM (36.3 mg/mL) were hydrolyzed in 0.1 N HCl at 100 °C for 60 min. Free D-galactose and D-glucose were resolved by descending paper chromatography using Whatman No. 1 paper in a mixture of *n*-butanol/pyri-

dine/0.1 N HCl (50/30/20) for 12 h [12,13]. Hydrolysis products were located by an aniline-phthalate reagent [20] and identified using D-galactose, D-glucose, L-fucose, *N*-acetylneuraminic acid (Type IV-S), intact lactose, raffinose or porcine submaxillary mucin as standards.

3. Results

3.1. Specificity and characteristics of the NAD(P)$^+$ dependent dehydrogenase enzymes

Fig. 1 shows the absorption spectra of the complex resulting from the addition of Cu-BCS reagent to a reaction mixture containing 90 nmol D-galactose with galactose dehydrogenase and NAD^+. The maximal absorption did not differ significantly with NADPH formed from the oxidation of D-glucose with glucose dehydrogenase (data not shown). Both NADH and NADPH reacted with the Cu-BCS reagent and produced an orange complex with a maximal absorption at 486 nm. No significant increase in absorbance at 486 nm was observed when Cu-BCS, $NAD(P)^+$, sugar, or dehydrogenase enzyme were each omitted from the incubation mixture, indicating that each of the above components were required for the color reaction to occur. Each of the dehydrogenases also proved specific for its substrate and formed a color product with either D-galactose or D-glucose. Enzyme specificity for substrate was evaluated using D-mannose, L-fucose, *N*-acetylglucosamine, lactose and sucrose as the test sugars. No significant increase in absorbance with any of these substrates indicated the specificity of galactose and glucose dehydrogenase with D-galactose or D-glucose only.

3.2. pH activity characteristics of glucose and galactose dehydrogenase enzymes

Glucose and galactose dehydrogenase each yielded a pH activity profile with an optimum at pH 8.5; i.e., with D-glucose and D-galactose serving as the respective substrates. In each instance, a pH of 8.5 was found optimal regardless of whether the monitoring for NAD(P)H production was performed directly (i.e., at 340 nm) or indirectly (i.e., at 486 nm by the Cu-BCS reagent). At 340 and 486 nm, the relative pH activity profiles (i.e., from the pH range of 6.0–9.5) for each enzyme were super-imposable (data not shown).

3.3. Sensitivity of colorimetric measurement of D-galactose and D-glucose

The Cu-BCS assay was able to detect the presence of 2.5 nmol of D-galactose or D-glucose and the absorbance was linearly proportional to exogenously added sugar from 2.5 to 250 nmol (0.833–83.3 μM). When equivalent and varying amounts of D-galactose or D-glucose were assayed, the absorption generated from each did not differ significantly, demonstrating that the oxidation of 1 nmol of sugar ultimately resulted in the production of 1 nmol of NAD(P)H. The standard curves of D-galactose (data not shown) and D-glucose (Fig. 2) were superimposable and each yielded a molar absorptivity of $2.36 \times 10^4\ M^{-1}\ cm^{-1}$.

3.4. Measurement of D-galactose and D-glucose in acid hydrolyzed lactose

Table 1 compares the amounts of D-galactose and D-glucose in lactose as detected by the Cu-BCS assay at 486 nm following hydrolysis of the disaccharide in 0.2 N HCl. A molar extinction coefficient of 23,600 was used in the determination of each sugar.

3.5. Quantification of D-galactose/D-glucose in PNP substrates

Table 2 shows that under the conditions of the assay and at different time intervals, equal amounts of D-glucose and *p*-nitrophenol were observed in the solution. The concentrations of *p*-nitrophenol and D-glucose were determined at 400 and 486 nm using a molar extinction coefficient of 18,100 [12] and 23,600, respectively. A plot of *p*-nitrophenyl-α-D-galactopyranoside hydrolysis (i.e., with 0.2 N

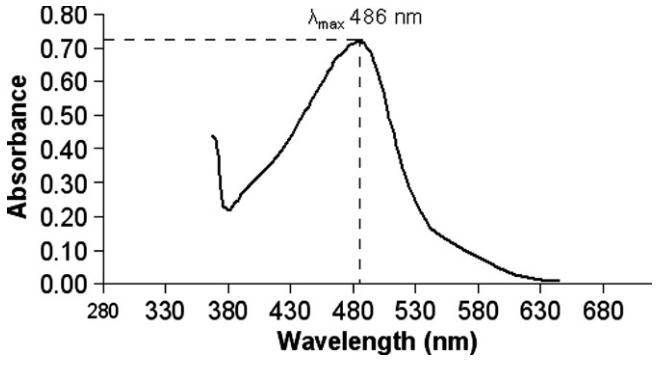

Fig. 1. Absorption spectrum of the orange complex formed from the addition of 1.5 mL of the Cu-BCS reagent to a 1.5 mL solution containing 90 nmol D-galactose, 0.860 μmol NAD^+, 0.1 U galactose dehydrogenase and 10 mM Tris buffer, pH 8.5, following incubation at 25 °C for 90 min.

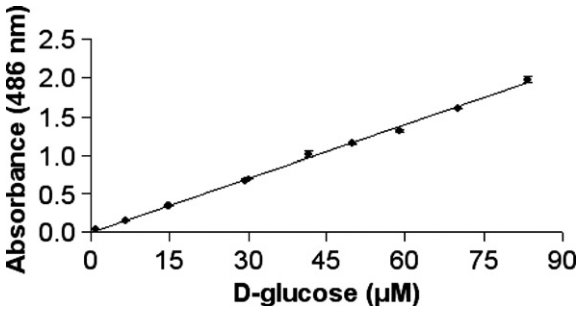

Fig. 2. Sensitivity of colorimetric measurement of Cu-BCS complex from the oxidation of D-glucose by glucose dehydrogenase. Each point represents an average of triplicate measurement, and all triplicate measurements for each point were within 5%.

Table 1
Comparison of the Amounts of D-Galactose and D-Glucose Released from Lactose[a]

D-Galactose (nmol)	D-Glucose (nmol)	Difference (%)
245	244	0.4
155	160	3.1
78	81	3.7
21	22	4.5

[a] All values represent an average of triplicate determinations, and all triplicate measurements for each value were within 5%.

Table 2
Amounts of *p*-nitrophenol and D-glucose released from PNP-α-D-glucopyranoside with time[a]

Time (min)	*p*-Nitrophenol (nmol)	D-Glucose (nmol)	Difference (%)
10	16.2	16.8	3.6
15	28.1	28.9	2.8
20	38.5	38.6	0.26
25	52.0	51.5	0.96
30	58.2	56.9	2.2

[a] All values represent an average of triplicate determinations, and all triplicate measurements for each value were within 5%.

HCl) yielded a similar profile to that of the *p*-nitrophenyl-α-D-glucopyranoside hydrolysis with time (data not shown).

3.6. Hydrolysis of D-galactose from porcine submaxillary mucin and raffinose by acid

Fig. 3 shows a plot of the time course hydrolysis of PSM (2.5 mg/mL) and raffinose (0.74 mg/mL) with 0.5 N HCl. The rate of D-galactose released from PSM was relatively constant over the first 60 min of incubation. After 75 min, no further increase in absorbance was observed and the quantity of D-galactose in solution reached a constant value of 250 nmol, indicating that all D-galactose from PSM was hydrolyzed. Hydrolysis of D-galactose from raffinose was linear for the first 30 min and then plateaued, indicating that most of the D-galactose in raffinose was hydrolyzed. At 30 min, 192 nmol of D-galactose was detected in the reaction mixture and this amount increased to a final value of 250 nmol after 75 min.

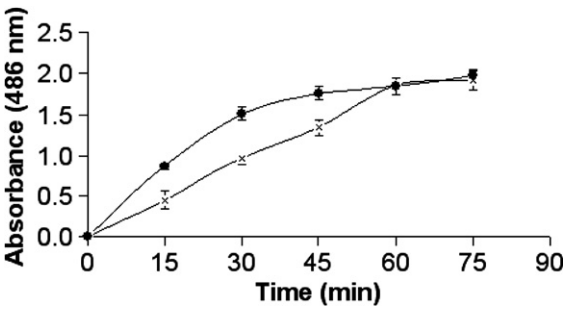

Fig. 3. Acid hydrolysis of D-galactose from raffinose (-■-) and porcine submaxillary mucin (-×-) with time. Each point represents an average of triplicate measurement, and all triplicate measurements for each point were within 5%.

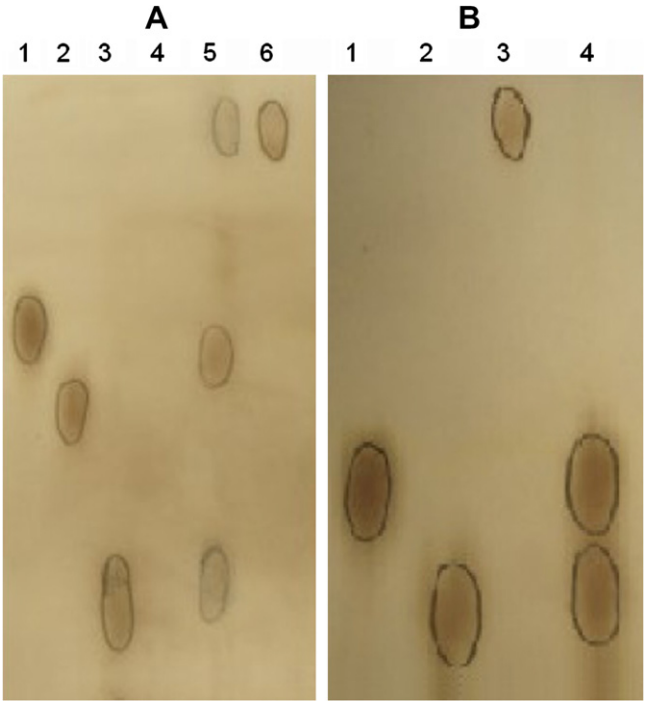

Fig. 4. (A) Identification of PSM hydrolysis products by descending paper chromatography. 1, D-galactose; 2, D-glucose; 3, L-fucose; 4, intact PSM; 5, hydrolyzed PSM; 6, *N*-acetylneuraminic acid (B) identification of raffinose hydrolysis products by descending paper chromatography. 1, D-galactose; 2, D-glucose; 3, intact raffinose; 4, hydrolyzed raffinose.

3.7. Identification of products released from lactose, raffinose and porcine submaxillary mucin by acid

Hydrolysis products of lactose, raffinose and PSM were verified by descending paper chromatography as previously described. Controls included D-galactose, D-glucose, L-fucose, *N*-acetylneuraminic acid, intact raffinose and intact PSM. Hydrolyzed PSM produced positive aniline–phthalate spots with R_f values that corresponded to the R_f values of D-galactose, *N*-acetylneuraminic acid and the L-fucose controls (Fig. 4A). The hydrolysis products of lactose (data not shown) and raffinose (Fig. 4B) produced positive aniline–phthalate spots that coincided with the D-galactose and D-glucose controls. No aniline–phthalate spots were detected for intact PSM; however, intact lactose and raffinose reacted with the aniline phthalate reagent and each yielded a spot that was outside the range of the D-galactose or D-glucose controls. It is speculated that the pronounced spots with intact lactose and raffinose were due to the aniline–pthalate reagent reacting with the unhydrolyzed sugars. No further testing was performed to confirm this speculation.

4. Discussion

Of the various dehydrogenase assays used for analyzing monosaccharides in glycoconjugates, the direct and indirect measurement of NAD(P)H by UV, fluorometric, and colorimetric methods have received considerable attention

[1,2,11–13]. A major reason for the popularity of the direct UV and the fluorometric methods has been due to their high sensitivity [2,13,16]. However, since these techniques do not provide for a visually detectable color change, they cannot be used in instances where qualitative analysis of glycoconjugates is desired. Additionally, the use of fluorometry as an analytical tool is limited by a dependence upon environmental conditions [1,12,16].

While the colorimetric measurement of NAD(P)H has overcome many of these difficulties, the performance of these assays is variable and so is the sensitivity between the different methods. A number of tetrazolium salts have been successfully employed in the measurement of glucose and galactose dehydrogenase enzymes [1,17]. Many of these tetrazolium reagents have been shown to be stable at room temperature, and once reduced by NAD(P)H, to form intensely colored formazans [1,17,18]. The transfer of electrons from NAD(P)H to tetrazolium salts can occur nonenzymatically in the presence of phenazine methosulfate [17,18] or can be catalytically induced by enzymes such as diaphorase [1]. While used routinely in dehydrogenase assays, phenazine methosulfate is not stable in the presence of light [19].

We have described here a novel assay for the determination of D-glucose and D-galactose in glycoconjugates that avoids the limitations of the direct UV and fluorometric assays and which requires no phenazine methosulfate or diaphorase. This assay is based on the reduction of Cu^{2+} to Cu^{+} in the presence of NAD(P)H and on the subsequent reaction of Cu^{+} with BCS to form a color complex. The Cu-BCS reaction was shown to be simple and rapid and to allow for the accurate determination of D-galactose and D-glucose in a number of compounds. When evaluated for its sensitivity, this method was able to detect as low as 0.833 ± 0.002 μM of D-glucose and 0.833 ± 0.005 μM of D-galactose with the absorbance being linearly proportional to exogenously added sugar from 0.833 to 83.3 μM (± 0.048 and ± 0.018 μM for D-glucose and D-galactose, respectively). With minor modifications, this assay can be applied for the determination of other monosaccharides whose oxidation can be achieved by specific $NAD(P)^{+}$ dependent dehydrogenases.

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