Clinical Application of the Serum 1,5-Anhydroglucitol Assay Method Using Glucose 3-Dehydrogenase

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We attempted to develop a novel serum 1,5-anhydroglucitol (1,5-AG) assay kit using glucose 3-dehydrogenase (G3DH) from *Halomonas* sp. α -15 strain. The major advantages of this method are that the 1,5-AG detection requires a very small amount of G3DH, and the enzyme catalyzes a simple reaction. The analytical performances were acceptable for clinical use

operated with a clinical automatic analyzer. The correlation with a commercial assay kit against sera of healthy volunteers was $y=0.975x+0.008$, $r=0.993$, $Sylx=1.32$ μ g/mL. However, sham-negative specimens were observed in the validation of this method using specimens from hospital patients. J. Clin. Lab. Anal. 16:299–303, 2002. © 2002 Wiley-Liss, Inc.

Key words: 1,5-anhydroglucitol; glucose 3-dehydrogenase; clinical analysis; validation

INTRODUCTION

1,5-anhydroglucitol (1,5-AG) is the 1-deoxy form of glucopyranose and is present as one of the constitutive sugar components in blood (1). Serum 1,5-AG concentration is normally stable at about 20–40 μ g/mL, but is greatly reduced to 0.5–10.0 μ g/mL in hyperglycemia. Since the serum 1,5-AG level correlates clearly with the serum glucose level, and the change is sensitive (2–4), it is generally used as a clinical marker of glycemic control in diabetes mellitus patients.

Various biochemical methods for 1,5-AG diagnosis have been developed, with pyranose oxidase-based systems currently being the most widely utilized for clinical analysis (5,6). Since pyranose oxidase is not selective toward 1,5-AG, pretreatment of serum specimens is necessary to remove glucose, which is another substrate of the enzyme. Detection of pyranose oxidase activity also requires combination with peroxides (horseradish) to catalyze the color-developing reaction. Furthermore, the effect of dissolved oxygen is not negligible in the enzymatic measurement system utilizing oxidases.

We previously reported the isolation of glucose 3-dehydrogenase (G3DH) from the marine bacterium *Halomonas* sp. α -15 strain. The enzyme catalyzes the oxidation of the C-3 hydroxyl group of various saccharides, such as 1,5-AG, glucose, methyl- α -D-glucopyranoside, maltose, and sucrose. We also showed

that the combination of G3DH and the fluorescent electron acceptor AlamarBlue™ (BioSource, Camarillo, USA) enabled 1,5-AG quantification within the range of 4.0–320 μ g/mL, which covers both the normal and diabetic blood 1,5-AG content (7). As this enzyme is a dehydrogenase and does not utilize oxygen as the electron acceptor, the reaction is not affected by dissolved oxygen. The 1,5AG-dependent G3DH activity can be measured by a simple color-developing reaction in combination with appropriate artificial electron acceptors. Furthermore, G3DH is highly reactive toward 1,5-AG, with a relative activity 72% of that with glucose (7). In contrast, pyranose oxidase reacts with 1,5-AG with a relative activity of only 19% compared with glucose (5). These advantageous features of G3DH encouraged us to develop a novel enzymatic method for the determination of 1,5-AG in serum.

Herein we report the construction and validation of a novel serum 1,5-AG assay kit, which uses G3DH and a tetrazolium salt as an electron acceptor, and is operated

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by a clinical automatic analyzer. As depicted in Fig. 1, the assay scheme consists of a combination of two reactions: 1) the elimination of glucose, maltose, and sucrose; and 2) the quantification of 1,5-AG.

MATERIALS AND METHODS

Chemicals

1,5-AG was purchased from Toronto Research Chemicals, Inc. (North York, Canada). G3DH was prepared from a culture of *Halomonas* sp. α -15 strain as previously reported (7,8). The specific activity of G3DH was approximately 5.0 U/mg of protein. The following chemicals were used: hexokinase (from recombinant yeast; Roche Diagnostics, Basel, Switzerland), pyruvate kinase (from rabbit muscle; Roche Diagnostics, Basel, Switzerland), α -glucosidase (from microorganism; TOYOBO, Osaka, Japan), β -fructosidase (from yeast; Roche Diagnostics, Basel, Switzerland), ascorbate oxidase (from *Acremonium* sp.; Asahikasei Industrial Co., Tokyo, Japan), disodium salt of adenosine 5'-triphosphate (ATP-2Na; Oriental Yeast Co., Tokyo, Japan), phosphoenolpyruvate-monocyclohexylammonium (PEP; Oriental Yeast Co., Kumamoto, Japan), and water soluble tetrazolium-1 (WST-1, Dojindo, Japan). The clinical assay kit used for 1,5-anhydroglucitol (1,5-AG) was Lana[®] 1,5AG Auto (Nihonkayaku Co., Tokyo, Japan) (6).

Clinical Serum Specimen Collection

Clinical serum specimens were kindly provided by Dr. A. Matsuoka (Hospital of Hyogo College of Medicine, Nishinomiya, Japan). Sera were collected from patients (n=278) at random and from healthy

volunteers (n= 90), with their signed consent for this study.

Construction of 1,5-AG Assay Kit Using G3DH

The sugar-eliminating process (reaction 1) and 1,5-AG-quantification process (reaction 2) were driven by reagents 1 and 2, respectively. Reagent 1 consisted of 5.0 KU of hexokinase, 10 KU of pyruvate kinase, 10.0 KU of α -glucosidase, 30.0 KU of β -fructosidase, 0.5 KU of ascorbate oxidase, 5.0 mmol of ATP-2Na, 10.0 mmol of PEP, 3.0 g of Triton X-100, and 1.0 mmol of WST-1 per liter of phosphate buffer (50 mmol/L, pH 7.0). Reagent 2 consisted of 5.0 KU of G3DH per liter of the same buffer.

The assay was carried out with a Hitachi 7150 automatic analyzer (Hitachi, Japan). The serum specimen (10 μ l) and reagent 1 (240 μ l) were combined into the reaction cuvettes and incubated for 5 min at 37°C. Reagent 2 (80 μ l) was then added and the absorbance change was measured for 5 min at 450 nm and 700 nm (main wave length and sub wave length, respectively). The 1,5-AG concentration was calculated from the calibration curve based on the absorbance of the 1,5-AG standards (two standards were respectively 0 μ g/ml and 30 μ g/ml of 1,5-AG concentration).

Evaluation of Analytical Performance

The linearity, analytical recovery rate, dilution linearity, interference of endogenous substances, reproducibility, and sugar (glucose, maltose, and sucrose) eliminating performances were tested. Serum specimen containing up to 20 g/L of glucose or 2 g/L of maltose or sucrose were measured by the G3DH method in order to evaluate the efficiency of the sugar elimination.

Validation of the G3DH Method by the Correlation Test

The correlations between the G3DH method and Lana[®] 1,5AG Auto (Nihon-Kayaku Co., Japan) were separately determined for the hospital patients' and healthy subjects' sera.

RESULTS AND DISCUSSION

Optimization of G3DH Reaction

Increasing the amount of G3DH added to 10–40 μ g/mL of 1,5-AG standards resulted in an increase in the rate of the color-developing reaction (Fig. 2A). Since a maximum was observed over 1.2 KU/L of G3DH, 5.0 KU/L was chosen as the quantity to use in reagent 2. The 1,5-AG assay kit using pyranose oxidase was reported to require 33 KU/L of enzyme for the

Reaction 1 (sugar eliminating process)

Reaction 2 (1,5-AG quantification process)

Fig. 1. Reaction scheme of 1,5-AG measurement in serum specimen.

Fig. 2. Effect of (A) G3DH and (B) WST-1 concentration on the color-developing reaction. The 1,5-AG standards, 0 $\mu\text{g/mL}$ ($\circ$), 10 $\mu\text{g/mL}$ ($\bullet$), 20 $\mu\text{g/mL}$ ($\blacktriangle$), 40 $\mu\text{g/mL}$ ($\blacksquare$), and 60 $\mu\text{g/mL}$ ($\blacklozenge$) were measured.

measurement of serum 1,5-AG (6). This more than 27.5-fold greater amount of enzyme required may be due to the lower relative activity of pyranose oxidase toward 1,5-AG, compared with G3DH (6,7).

We then investigated the ideal concentration of electron acceptor (WST-1) to use in the 1,5-AG assay method. The color development rate increased with increasing WST-1, reaching a maximum over 1.0 mM (Fig. 2B). The quantity of WST-1 to use in reagent 1 was therefore chosen to be 1.0 mM.

Analytical Performance of the 1,5-AG Quantification Process

With the reaction mixture optimized as above, the analytical performance of reaction 2 was evaluated.

The linear range of the calibration curve was observed up to 60 $\mu\text{g/mL}$ of 1,5-AG, which covers the range found in sera (Fig. 3). The linear regression formula of absorbance (y) vs. 1,5-AG concentration (x) was $y = 4.18x + 14.17$ mAbs, $r = 0.999$, $Sy|x = 3.37$ mAbs. The detection limit of the method, defined as the concentration corresponding to the value 3 SD above the mean for the zero calibrator, was 0.5 $\mu\text{g/mL}$ of 1,5-AG. The precision (CV), as determined by replicate analyses of three sera ($n = 10$), was 0.72–0.85%, which is considered acceptable for clinical analysis. The analytical recovery was tested by the addition of three concentrations of 1,5-AG to three sera. The recovery rates were ascertained to be 99.6–104.0%, and were thus acceptable for clinical use (Table 1). Dilution linearity was determined by analyzing five data points from diluted sera ($n = 3$). In each case, plotting the results produced straight lines passing through the origin.

Analytical Performance With Potential Interfering Substances

Reagent 1 is composed of several enzymes in order to eliminate possible substrates of G3DH existing in the serum specimen. Glucose is eliminated by hexokinase, combined with an ATP recycling reaction driven by pyruvate kinase and excess PEP. α -glucosidase and β -fructosidase are used for maltose and sucrose degradation, respectively, and the resulting glucose is eliminated as above. In the incubation with reagent 1, glucose contained in serum is converted to glucose-6-phosphate, with which G3DH does not react. The presence of 6 g/L of glucose in serum, which is the maximum value observed in hyperglycemia, resulted in a slight G3DH color-developing reaction with WST-1, below the defined detection limit. Maltose or sucrose up to 0.2 g/L also failed to produce a signal above the detection limit of this assay. Reagent 1 therefore very effectively fulfills its role in eliminating interfering sugars.

Other serum components that could potentially interfere with the assay were also tested at physiological concentrations. Free bilirubin up to 180 mg/L, conjugated bilirubin up to 220 mg/L, intralipid up to 0.2%, hemoglobin up to 5 g/L, and ascorbic acid up to 1 g/L did not affect the 1,5-AG assay results obtained by the G3DH method.

Validation of the G3DH Method

The clinical application of the G3DH method was evaluated using serum specimens from hospital patients and healthy volunteers by correlation tests against the conventional 1,5-AG assay method based on pyranose oxidase (Lana[®] 1,5AG Auto; Nihon-Kayaku Co.,

Fig. 3. Calibration curve of the G3DH method.

TABLE 1. Analytical recovery of 1,5-AG added to serum

Added	1,5-AG concentration (μg/mL)		
	Serum A (%)	Serum B (%)	Serum C (%)
Original	5.5	12.0	14.0
4.4	10.3 (104.0)	16.8 (102.4)	18.5 (100.5)
8.9	14.9 (103.5)	21.4 (102.4)	22.8 (99.6)
13.4	19.1 (101.1)	25.8 (101.6)	27.6 (100.7)

Recovery rate (%) is shown in parentheses.

Japan). The evaluation using specimens from the healthy subjects showed that the G3DH method (y) correlated well with the pyranose oxidase method (x) ($y=0.975x+0.008$, $r=0.993$, $Sy|x=1.32$ μg/mL; Fig. 4A). However, with the specimens from the patients' group, some discrepancies of assay value between the G3DH method (y) and the pyranose oxidase method (x) were observed. The linear regression was $y=0.905x+3.080$, $r=0.941$, $Sy|x=3.44$ μg/mL (Fig. 4B). The G3DH method resulted in higher 1,5-AG concentrations than those determined by the pyranose oxidase method in all of the discrepant specimens. Hyperglycemia is diagnosed by the reduction of serum 1,5-AG concentration (cut off: 13.0 μg/mL) (2–4). Due to the overestimation of 1,5 AG concentration by the G3DH method, the specimen which should be diagnosed as hyperglycemia would be misdiagnosed as a normal healthy subject. Such a misdiagnosis, in

Fig. 4. Correlation tests between the conventional pyranose oxidase assay and the G3DH assay. Comparisons were made between the two assays sera from (A) healthy volunteers ($n=90$) and (B) hospital patients ($n=278$).

which positive (hyperglycemia) is diagnosed as negative (healthy), is categorized as sham-negative.

The sham-negative results were not caused by the presence of serum proteins, which might affect the results, as deproteinization (addition of 5% trichloroacetic acid) of the serum did not resolve the discrepancy (data not

shown). The sham-negative specimens did not contain any compounds that might inhibit enzymes used in the sugar-eliminating processes. It also was not causative, as the addition of glucose, maltose, and sucrose to these sera did not result in increased assay values (data not shown). These sham-negative specimens may therefore contain G3DH substrates that may not be eliminated by the sugar-eliminating processes. A characteristic property of G3DH is its broad substrate specificity. G3DH can oxidize most glucoside compounds, with Michaelis constants (K_m) ranging from 10^{-3} to 10^{-2} M (7). We previously reported the presence of N-carbamoyl- β -D-glucopyranosylamine (NCG) in human serum (9). Although the mechanism of NCG formation, and its physiological significance, remain unknown, NCG is believed to be produced in vivo, as it has not been utilized in medicine or food. Considering that NCG was found to be a substrate of G3DH, the sham-negative sera might contain considerable amounts of NCG, thus resulting in the discrepancy observed between the two methods. Therefore, clinical application of the G3DH method should be further needed for the development of the elimination process of causative substrate, e.g., NCG, or the sorting out of the sham-negative specimens from the patient group would be available for the clinical application.

The enzymatic elimination process of the causative substrate can be developed by the identification of the substrate. If NCG is the causative substrate, it can be hydrolyzed by the enzyme reported in the existence in *Clostridium innocuum* (10). Further enzymatic studies will provide us with an enzymatic elimination process for NCG. On the other hand, the fact that the sham-negative appeared only in the hospital patients suggests that there is a relationship between the sham-negative and disease and/or therapy. If the presence of a causative substrate is due to a specific disease and/or therapy, a specimen from a patient with the specific disease may be distinct in the G3DH assay.

Further studies concerning the identification and distribution of the causative substrate are essential

in order to apply the G3DH method clinically for the serum 1,5-AG assay.

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