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A Novel Fully Enzymatic Method for Determining Glucose and 1,5-Anhydro-D-Glucitol in Serum of One Cuvette

Feng Jing · Lu Jun · Yuping Wang · Mingan Zhu · Zhang Yong · Xie Fei · Jicai Zhang

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Abstract The aim of the study was to set up a novel fully enzymatic method for screening glucose and 1,5-anhydro-D-glucitol (1,5-AG) in one cuvette. We have determined glucose and 1,5-AG, based on glucokinase (GK) converting glucose to G6P, a compound that can be catalyzed ultimately into 6-PGA by G-6PD and its coenzyme $NADP^+$, and then calculated glucose concentration according to absorbance variety. Furthermore, pyranose oxidase was used to oxidize 1,5-AG with the formation of 1, 5-anhydro-fructose and H_2O_2. Measurement was done according to Trinder's reaction principle. The mean within-run and day-to-day precision (CV) of this method for glucose was 0.88% and 1.4%, and also that for 1,5-AG was 1.05% and 1.94%, respectively. The mean recovery rate of two targets was 100.2% and 101.6%, respectively. The correlation (R^2) between the results of 1,5-AG obtained with our proposed method (y) and those obtained with LanaAG method (x) was 0.999 (y=1.002x−0.675 μmol/l; n=86), and the correlation (R^2) of glucose between the results obtained with our GK method (y) and those obtained with recommendatory hexokinase method (x) was 0.9999 (y=1.0043x+0.1229 mmol/l; n=86). The reference range (95%) of serological glucose and 1,5-AG was 3.7 to 5.7 mmol/l (4.70±0.51 mmol/l) and 83.1 to 240.7 μmol/l (161.9±40.2 μmol/l), respectively; and there was no difference with age and sex (P>0.05). This newly developed method was dependable and steady-going, with analysis automatization, and allows quicker and easier measurement of serum glucose and 1,5-AG in one identical reaction cuvette in-phase than previously described methods.

Keywords Glucose · 1,5-Anhydro-D-glucitol · Assay

Abbreviations

1,5-AG	1,5-anhydro-D-glucitol
PROD	pyranose oxidase
GK	glucokinase
G6P	glucose 6-phosphate

F. Jing (✉) · L. Jun · Y. Wang · M. Zhu · Z. Yong · X. Fei · J. Zhang
Department of Laboratory Medicine Center of Taihe Hospital, Yunyang Medical College, 442000 Shiyan, China
e-mail: fengjinggood@hotmail.Com

HRP	horseradish peroxidase
G-6PD	glucose-6-phosphate dehydrogenase
HTIB	3-hydroxy-2,4,6-triiodobenzoic acid
4-AAP	4-aminoantipyrine
TG	triglyceride
Hb	hemoglobin
T bil	total bilirubin
HK	hexokinase

Glucose and 1,5-anhydro-D-glucitol (1,5-AG) are uppermost six-carbon polyol in human serum. The latter substance that is also called 1-deoxyglucose is a 1-deoxy form of glucopyranose, one of the principal polyols in human cerebrospinal fluid and serum [1–2]. The 1,5-AG excretion rate is increased in periods of glucosuric hyperglycemia, apparently due to competition for reabsorption for hyperglycemia [3–4]. Poor glycemic control can therefore be accompanied by a significant overall decrease in 1,5-AG. In principle, serial 1,5-AG monitoring on appropriate time scales can be used to monitor the success of intervention in diabetes, as evidenced by reestablishment of a euglycemic state, or to indicate episodic hyperglycemia, as evidenced by decreases in 1,5-AG or an alteration in the rate of repletion of 1,5-AG to normal values [5–6]. Given the established principle of close monitoring of glucose and GHb as standards of care in diabetes [7], further study of the information provided by 1,5-AG monitoring in diabetes care is certainly warranted. The overwhelming majority of data on clinical use of 1,5-AG comes from Japan where reduced concentrations of 1,5-AG in serum of hyperglycemic patients in comparison to euglycemic subjects have been demonstrated. Additionally, a gradual normalization of 1,5-AG values for patients responding to anti-diabetic therapies [8] has been demonstrated, and studies have shown that 1,5-AG measurements reflect glycemic status over the previous 48 h to 2 weeks. The early research studies of 1,5-AG employed measurement via chromatographic methods [9–10]. With either method, it was necessary to remove plasma proteins and glucose prior to measurement of 1,5-AG. And pyranose oxidase (PROD; EC 1.1.3.10) can catalyze the oxidation of many sugars and sugar alcohols with pyranose structure, such as 1,5-AG and glucose [11]. Hereby, to measure 1,5-AG with PROD, the glucose needs to be removed beforehand with a column such as the LanaAG kit [11–12].

Based on prior studies, we have developed a novel fully automated assay of glucose and 1,5-AG based on enzymatic reactions in one cuvette. Our assay utilizes GK, which converts glucose to G6P, a compound that can be catalyzed ultimately into 6-PGA by G-6PD and its coenzyme nicotinamide adenine dinucleotide phosphate (oxidized form; $NADP^+$), which does not react with PROD, and then calculates glucose concentration according to absorbance variety. Furthermore, PROD was used to oxidize 1,5-AG with the formation of 1, 5-anhydro-fructose and H_2O_2. Measurement was done according to the Trinder's reaction principle (Fig. 1). Here we introduced the new method of assay.

Materials and Methods

Materials

Reagents 1,5-AG, $NADP^+$ and G6PD were purchased from Sigma Chemical, St. Louis, MO, USA. PROD (from *Polyporus obtusus*; specific activity for glucose, 13.0 kU/g of

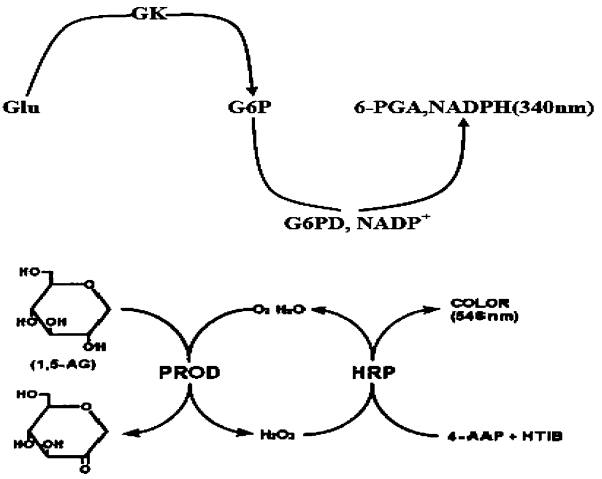

Fig. 1 Reaction scheme for determining 1,5-AG and glucose. Abbreviations are defined in text footnote

protein) was from Takara Shuzo, Kyoto, Japan; GK (from *Bacillus stearothermophilus*; specific activity, 350 kU/g of protein) was from Unitika, Osaka, Japan; $MgCl_2$ was from Boehringer Mannheim Diagnostica, Mannheim, Germany; horseradish peroxidase (HRP; EC 1.11.1.7) and ascorbate oxidase (EC 1.10.3.3) from *Cucumis* were from Toyobo, Osaka, Japan; 4-aminoantipyrine (4-AAP) and all remaining reagents were obtained from Wako Pure Chemical Industries, Osaka, Japan.

Reaction reagents Reagent 1 (R1) consisted of 6.0 kU of GK (final concentration in reaction cuvette, 2.9 kU/l), 6.0 mmol/l of $NADP^+$ (final concentration in reaction cuvette, 2.9 mmol/l), and 15 mmol of $MgC1_2$ per liter of Tris–HCl buffer (50.0 mmol/l, pH 7.4); reagent 2 (R2) consisted of 16 kU/l of G6PD (final concentration in reaction cuvette, 8 kU/l), 4.0 mmol of adenosine triphosphate (ATP), and 18 kU of HRP per liter of Tris–HCl buffer (50.0 mmol/l, pH 7.4); reagent 3 (R3) consisted of 90 kU of PROD (final concentration in reaction cuvette, 29.3 kU/l), 1.5 mmol of 4-AAP, 5.0 kU of ascorbate oxidase, and 4.5 mmol of 3-hydroxy-2,4,6-triiodobenzoic acid (HTIB) per liter of 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer (200 mmol/l, pH 7.6).

Apparatus The RA 1000 (Technicon, American) and Hitachi 7080 automated analyzer (Hitachi, Japan) were used.

Samples Serum samples were obtained from 120 cases of healthy individuals and 61 cases type 2 diabetic patients (diagnostic with diabetic time 1 week to 2 years, mean 6.5 months, without any microangiopathy and kidney pathological changes) in our hospital and used in accuracy and comparison studies. Serum was separated from peripheral venous blood by centrifugation within 30 min after collection, stored at 4°C, and analyzed within 1 week. The procedures followed were in accordance with the Helsinki Declaration of 1975, as revised in 1983.

Hexokinase Method Briefly, glucose and ATP are catalyzed to G6P and ADP by hexokinase (HK), and G6P was further generated to 6-PGA and with $NADP^+$ to $NADPH+H^+$ by G6PDH.

Assay Procedure The assay is based on an endpoint method determination principle. This is a highly specific method for determining the concentration of glucose in serum or plasma by spectrophotometrically measuring the NADP formed from HK catalyzed transformations of glucose and various intermediates. Glucose calibrator solutions (0 and 50 mmol/l) and samples were loaded into the appropriate positions along with R1; the assay settings for the RA1000 were entered, and the assay was started. The settings were as follows: wavelength, 340/405 nm; sample volume, 10 μl; R1, 150 μl; R2, 150 μl; reaction temperature, 37°C; calibrators, 50 mmol/l and saline (0 mmol/l). Initially, R1 and the sample were poured into the reaction cuvettes for preincubation of 5 min; shortly, R2 was added, and the absorbances A_1 (at time point no. 5) and A_2 (at time point no. 19) were read. After reacting for 14 min, the concentration of glucose was calculated from the absorbance change. Furthermore, wavelength was shifted to 540/700 nm, and 150 μl of R3 was added into reaction cuvettes, reading the absorbance A_3 just then (at time point no.20; Fig. 2). After reacting for 5 min, we read absorbance A_4 (at time point no.25). The concentration of 1,5-AG was calculated from the absorbance change. A linear calibration curve based on absorbance of the 0 and 550 μmol/l calibrators was used for this calculation. The calculation formulae are, briefly, as follows:

$$\begin{aligned}\text{Glucose(mmol/l)} &= (A_2 - A_1) \times \text{standard concentration} / (A_{\text{standard2}} - A_{\text{standard1}})\\ 1,5 - \text{AG(μmol/l)} &= (A_4 - A_3) \times \text{standard concentration} / (A_{\text{standard4}} - A_{\text{standard3}})\end{aligned}$$

Results

Optimization of Enzymes and Coenzyme Concentration

GK can convert glucose into G6P, and the latter is converted into 6-PGA further by G-6PD and $NADP^+$. We found that the concentration of GK was increased from 1.0 to 8.0 kU/l; even 50.0 mmol/l glucose in serum was completely converted to 6-PGA with GK of at least 1.0 kU/l. Thereafter, we used 2.0 kU/l as the final concentration in the reaction mixture. The concentration of G-6PD was varied from 1.0 to 10.0 kU/l, and $NADP^+$ varied from 0.5 to 2.9 mmol/l; we found that 50.0 mmol/l of glucose in serum was sufficiently converted with 7.5 kU/l of G-6PD, 1.8 mmol/l of $NADP^+$, therefore, we chose 8.0 kU/l of G-6PD and 2.0 mmol/l of $NADP^+$ as the final concentration to allow a margin of safety. With PROD

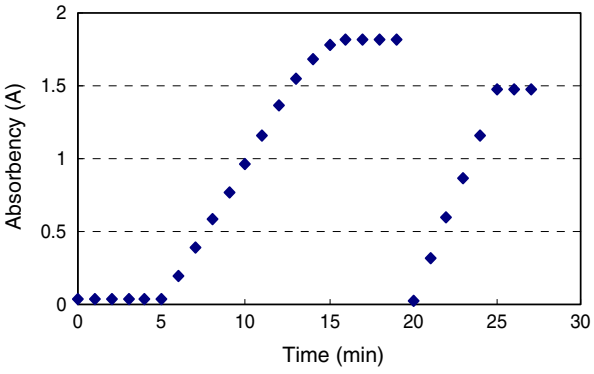

Fig. 2 Dynamical calibration curves of glucose and 1,5-AG. Calibration process of glucose on the *left of graph* at 0 to 19 points, and also calibration process of 1,5-AG on the *right of graph* at 20 to 25 points. A_1 was read at no. 5 min, A_2 was read at no. 19 min, A_3 was read at no. 20 min, and A_4 was read at 25 min

higher than 29.3 kU/l in the final reaction mixture, the difference in the absorbance of 550 μmol/l 1,5-AG reached a plateau. Therefore, we used this amount as the standard concentration in the reaction mixture, which corresponds to 90 kU/l in R3.

We examined the effects of kind of buffer and the concentrations of all the components involved in the enzyme reactions. To determine the suitable concentration of all the components, we varied the concentration of each component of R1 and R2 and investigated the potential effects of these changes on the measurement of serum glucose and 1,5-AG.

Calibration curve A serially diluted glucose (0 to 50.0 mmol/l) and 1,5-AG calibrator solution (0 to 550 μmol/l) were used to screen time vs. absorbance change of reaction process (Fig. 2). After R1 was preincubated for 5 min, the calibrator was then poured into reaction cuvettes for reacting of 14 min; 50 mmol/l glucose could be fully converted to 6-PGA during the process. What's more, 550 μmol/l 1,5-AG was also fully converted to 1,5-anhydro-fructose after injecting R3 into reaction cuvettes for reacting of 5 min. Therefore, our experimental parameter of glucose and 1,5-AG were framed according to calibration curve.

Analytical performance with serum samples Using serum samples from normal subjects, mixtures and patients, we determined the precision and linearity of measurement with our system, with interference by routine substances including triglyceride (TG), hemoglobin (Hb), and total bilirubin (T bil), and the correlation between measurements of glucose and 1,5-AG obtained with our system and with the HK and the Lana AG assay.

Precision and recovery The mean within-run mix serum composition of healthy and type 2 diabetes patients of glucose and 1,5-AG precision (CV) were 0.88% and 1.05%, and day-to-day precision (CV) were 1.40% and 1.94%, respectively (Table 1). These values were considered acceptable, and our methods were in fact superior to those obtained for the LanaAG and HK. We performed analytical recovery studies at three concentrations of glucose and 1,5-AG in serum samples from healthy volunteers and a diabetic patient. he results were good (mean 100.2% and 101.6%, respectively.) and acceptable for clinical use (Table 2).

Interference We studied potential interference from endogenous substances and additives, using mixed serum samples which include glucose (6.1 mmol/l) and 1,5-AG(122.5 μmol/l) from healthy volunteers and type 2 diabetic patients. We divided the mixed serum samples into three shares. One share was added an equivalent volume of TG (8.9 mmol/l), the second and third shares were joined equivalent volumes of Hb (4 g/l) and T bil (350 μmol/l), and the interferences of the above three substances to glucose and 1,5-AG were measured by our method. Interestingly, the interference rates for glucose was −0.32%, −1.48%, and 0.49%,

Table 1 Precisions of measurement of glucose and 1,5-AG (n=20).

	Glu (mmol/l)		1, 5-AG (μmol/l)	
	$\overline{X}_{\pm s}$	CV %	$\overline{X}_{\pm s}$	CV %
Within-run	2.21±0.03	1.35	14.7±0.25	1.70
	5.55±0.05	0.90	153.8±1.3	0.84
	15.9±0.07	0.40	455.5±2.8	0.61
Day-to-day	2.22±0.04	1.80	15.1±0.41	2.70
	5.49±0.08	1.46	154.5±3.1	2.00
	16.1±0.15	0.93	460.3±5.2	1.12

Table 2 Analytical recovery of glucose and 1,5-AG added to serum.

	Measurement	Joined	Recovery	Recovery ratio (%)
	Concentration			
1,5-AG (μmol/l)	183.2	61.0	60.7	99.5
	280.2	150.5	157.7	104.9
	728.3	602.0	605.8	100.6
Glu (mmol/l)	8.28	2.20	2.18	99.1
	11.70	5.55	5.56	100.2
	22.4	15.9	16.1	101.3

and 0.49%, 1.96%, and 1.39% for 1,5-AG, respectively. Therefore, the interference substances of normal level for TG, Hb, and T bil did not take effect on glucose and 1,5-AG's measurement if the absolute value of patient error was lower than 5%.

Comparison with other methods We compared the measurement outcome of our methods with LanaAG and HK methods; the regression equation for 1,5-AG values determined by the present method (y) and those by the Lana AG method (x) was $y=1.002x-0.675$ μmol/l ($n=86$, $R^2=0.999$), and regression equation for glucose values determined by our method (y) and those by the HK method (x) was $y=1.0043x+0.1229$ mmol/l ($n=86$, $R^2=0.9999$). Both degrees of correlation were satisfactory, and the intercept was close to 0 (Figs. 3 and 4).

Stability of reagents Reagents 1 and 2, were confected and encased in brown bottle and stored at 4°C for 10 weeks, and then we compared the results of glucose and 1,5-AG of the mixtures of serum samples with freshly made reagents. As expected, the absorbency did not change from 10 to 60 min after sample was mixed with reagents.

Normal reference range We measured serum glucose and 1,5-AG of 120 cases of health volunteers by this method. The concentration of glucose and 1,5-AG submitted normal distribution, and 95% reference range of serum glucose and 1,5-AG was 3.7 to 5.7 mmol/l (4.70±0.51 mmol/l) and 83.1 to 240.7 μmol/l (161.9±40.2 μmol/l), respectively. There was no difference with age and sex ($P>0.05$).

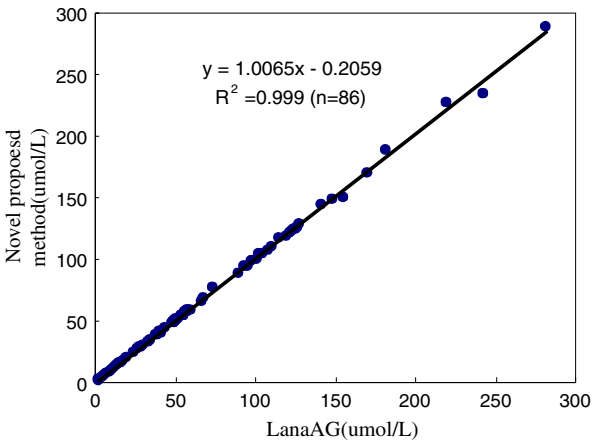

Fig. 3 Correlations between measurements obtained with the novel proposed method and the LanaAG method

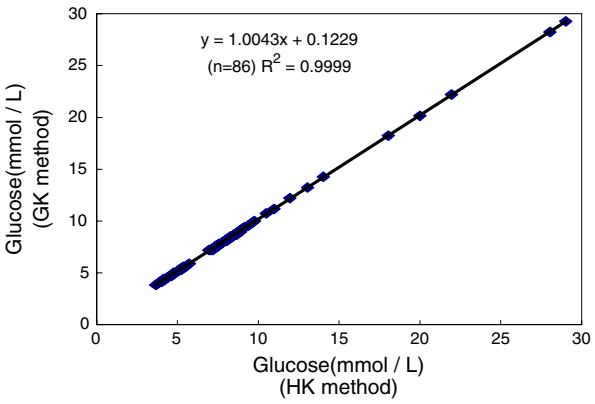

Fig. 4 Correlation between HK (*x*) and GK (*y*) methods for glucose measurements in diabetic patients and health controls

Discussion

1,5-AG is thought to originate mainly from orally ingested food [13] and is distributed to all tissues and organs. Because of a large pool in the body and its metabolic inertness, the concentration of 1,5-AG is stable and fluctuates within a remarkably narrow range in a normal human population [14]. There is little diurnal variation, and the concentration is not influenced by the intake of food or the glucose load [15]. The slow turnover of serum 1,5-AG observed in rat studies [16] implies that 1,5-AG is not an energy metabolite. The reduction of serum 1,5-AG concentrations in diabetic patients is mainly due to its accelerated urinary excretion, which is coincident with glycosuria [17]. Because of its low molecular mass, hydrophilic character, and unbound form, 1,5-AG is freely filtered through the glomerular basement membrane. The concentration of 1,5-AG is lower in urine than in plasma because of the participation of an active transport mechanism in the reabsorption process. Since 1,5-AG is competitively reabsorbed by a glucose transporter in renal proximal tubules, it is lost to urine when the tubular reabsorption capacity is overloaded by glycosuria. Thus, the degree of reduction of serum 1,5-AG depends simply on the urinary excretion of glucose. Recommended method to measure glucose is HK method in the world; also handy and credible methods of screening 1,5-AG were LanaAG and fully enzymatic reaction. We developed a novel method for screening serum glucose and 1,5-AG in-phase. The assay utilizes GK, which converts glucose to G6P, a compound that can be catalyzed into 6-PGA by G-6PD and its coenzyme $NADP^+$ at last, which does not react with PROD. And then, glucose concentration is calculated according to absorbance changes. Furthermore, after varying wavelength and second wavelength, PROD was used to oxidize 1,5-AG with the formation of 1, 5-anhydro-fructose and H_2O_2. Measurement was done according to Trinder's reaction principle. We set up a novel full enzymatic method based on high specificity and sensitivity for measuring glucose and 1,5-AG in-phase with automatization analysis. Our result showed that the method was provided with sensitivity, accuracy, stabilization, and specificity, and the linear ranges of glucose and 1,5-AG were competent for screening out and controlling therapy in diabetic patients.

We have optimized experimental condition of the enzyme reaction and pH of reaction system using HK instead of GK. However, some types of HK, e.g., from *Bacillus* spp. and *Saccharomyces* tpp., gave results slightly lower than GK measurements of 1,5-AG. We speculate that some 1,5-AG was involved in HK reaction system, which induced false decreasing of the concentration of 1,5-AG. We chose tri-hydroxymethy-aminomethane

hydrochloric acid (pH 7.4) as the buffer for measuring glucose to assure the largest enzymatic activity of GK, G-6PD, and coenzyme $NADP^+$. In succession, we confirmed the standard concentration of PROD to correspond to 29.5kU/l as the ultimate amount, which, firstly, had fully ensured about 550 μmol/l of 1,5-AG converted to 1,5-anhydro-fructose owing to its difficult derivation and yellowish color after confected liquor. Succulently, we studied the methodology of this method. The linear range of glucose was significant, satisfying from 0.2 to 40 mmol/l, which should be diluted and re-measured provided that the value is overrunning the range. Measurement range of 1,5-AG was 2.1 to 550 μmol/l, which is close to previous reported range [18]. The precisions and accuracies were up to the mustard of routine conditions variance (RCV); the within-means CV% of glucose and 1,5-AG were lower than 2%, and that of day-to-day were under 3%; and the mean recoveries for the targets were 100.2% and 101.7%, respectively. High TG, slight hemolysis, and conjugated bilirubin did not influence the measurement. The relativity of these methods to measure glucose and 1,5-AG compares beauty with HK and LanaAG methods. The 95% reference range of glucose was 3.7 to 5.7 mmol/l, and 1,5-AG was 83.1 to 240.7 μmol/l (161.9± 40.2 μmol/l), respectively.

The methods we developed were dependable and steady-going, with analysis automatization. However, it is hard to measure the two targets at one identical reaction cuvette in-phase practically because currently, many biochemistry analytical apparatus are programme-confined. And the methods for screening glucose and 1,5-AG would be an expansion in routine laboratory if the program of software had been liberated allodiality.

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