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Quantification of 1,5-anhydro-D-glucitol in urine by automated borate complex anion-exchange chromatography with an immobilized enzyme reactor

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Abstract

HPLC using a borate form of a strongly anion-exchange resin column and an immobilized enzyme reactor for colorimetric detection was used to quantify urinary 1,5-anhydro-D-glucitol. Urine samples were introduced into the system every 7 min without any pretreatment, and after separation of interfering substances in the column, 1,5-anhydro-D-glucitol was successively detected. Quantitative determination of urinary 1,5-anhydro-D-glucitol was possible within the 1.2–300 μmol/l range. The coefficient of variance was less than 3% and the correlation between results obtained with our system (y) and those obtained by gas chromatography–mass spectrometry (x) was $y=0.983x-1.287$ μmol/l ($n=42$, $r=0.998$).

Keywords: 1,5-Anhydro-D-glucitol

1. Introduction

1,5-Anhydro-D-glucitol (1,5-AG), a polyol with a pyranoid ring structure, is found at high concentrations in the serum of normal subjects and it decreases in patients with diabetes mellitus or renal disease [1–4]. Serum 1,5-AG has been used as a clinical marker of glycemic control in diabetic patients [5]. The reduction of serum 1,5-AG concentration in diabetic patients has been thought to be due to a competitive inhibition of its reabsorption at the renal proximal tubuli by a glucose transporter in the presence of excess glucose in the glomerular filtrate [6–8]. Under normal conditions, 1,5-AG is efficiently reabsorbed and the serum 1,5-AG concentration is maintained at high levels. When there is glucosuria, the urinary excretion of 1,5-AG increases temporarily and the serum 1,5-AG concentration decreases. However, data from other studies have suggested that 1,5-AG and mannose are reabsorbed in the renal tubuli by a common mechanism which is distinct from the main glucose transport system [9]. The mechanism of 1,5-AG reabsorption in the renal tubuli is still unclear. The determination of urinary 1,5-AG is very important in order to clarify the mechanism of 1,5-AG reabsorption.

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Urine 1,5-AG concentrations have been determined by GLC [9,10], gas chromatography–mass spectrometry (GC–MS) [6–8] and HPLC [11,12]. However, these methods require a series of sample pretreatments. In the case of GLC and GC–MS, an internal standard as well as partial purification (deproteinization and/or treatment with an ion-exchange resin), concentration and derivatization before the assay are also needed. In the case of HPLC systems, which consist of an OH^- form of anion-exchange or ODS column, immobilized pyranose oxidase (PROD) and an electrochemical detector, the sample has to be partially purified because PROD oxidizes several sugars and sugar-related substances in urine. To avoid the interference of glucose and other substances, urine samples are passed through a two-layer mini-column packed with an OH^- form of anion-exchange resin and a H^+ form of cation-exchange resin prior to HPLC.

Moreover, previous commercially available kits to determine serum 1,5-AG were not applicable to the determination of urinary 1,5-AG, because the concentrations of interfering substances found in urine are different from those present in serum. In the case of a manual enzymatic method [13], according to which serum glucose is removed using a two-layer mini-column and the eluted 1,5-AG is detected spectrophotometrically using PROD and horseradish peroxidase (HRP), the results are affected by urinary *myo*-inositol which passes through the mini-column and is oxidized by PROD. The fully enzymatic method developed by Fukumura et al. [14] is not suitable for the assay of urinary 1,5-AG either. In this method using glucokinase and PROD, the interfering glucose is converted to glucose 6-phosphate which is not catalyzed by PROD. However, glucokinase can not convert all interfering substances present in urine into compounds that do not react with PROD.

Recently, we developed a fully automated flow injection system suitable for quantifying serum 1,5-AG in a clinical setting [15]. This system also uses PROD and HRP, as shown in Fig. 1. Substances that would interfere with the enzymatic reaction are adsorbed by the clean-up column packed with a borate form of an anion-exchange resin and the eluate containing 1,5-AG is mixed with a color-

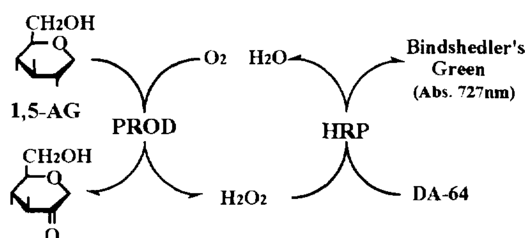

Fig. 1. Principle of the enzymatic determination of 1,5-AG.

developing solution containing N-(carboxymethylaminocarbonyl) - 4,4′ - bis(dimethylamino)-diphenylamine, sodium salt (DA-64) as the chromogen. The mixture is then introduced into the enzyme reactor packed with immobilized PROD and immobilized HRP, where 1,5-AG is oxidized and the resulting H_2O_2 reacts with DA-64 to produce Bindshedler's Green (λ_{max} 727 nm, ϵ=90 000), which is detected colorimetrically. In this system no pretreatment of serum is required because the borate form of the anion-exchange resin with a hydrophilic matrix has a huge capacity to adsorb interfering substances.

It is known that sugars and their related compounds are well separated by borate complex anion-exchange chromatography [16]. In this work we investigated the possible application of a flow injection system to the assay to determine urinary 1,5-AG without any pretreatment of urine samples.

2. Experimental

2.1. Materials and reagents

1,5-AG was purchased from Sigma (St. Louis, MO, USA). PROD (13 kU/g) and HRP (250 kU/g) were from Takara Shuzo (Kyoto, Japan) and Toyobo (Osaka, Japan), respectively. DA-64 was from Wako Pure Chemical Industries (Osaka, Japan). The anion-exchange resin AG-TOYOPEARL and gels TSKgel AF-Epoxy TOYOPEARL 650 and AF-Amino TOYOPEARL 650 were from Tosoh (Tokyo, Japan). Other reagents were obtained from commercial sources and were of analytical grade.

2.2. Separation column

The borate form of AG-TOYOPEARL, a strongly anion-exchange resin of the quaternary ammonium type with a hydrophilic matrix, was packed into a 120 mm×6 mm polyacetal resin column with 0.25 mol/l of boric acid. The packed column was stored at room temperature until use.

2.3. Enzyme reactor

PROD and HRP were immobilized to TSKgel AF-Epoxy TOYOPEARL 650 and TSKgel AF-Amino TOYOPEARL 650, respectively, and then packed into a polyacetal resin column that was divided into two parts with a filter, as previously described [15]. The upper part of the reactor (35 mm×3 mm) was packed with the immobilized PROD and the lower part (20 mm×2.5 mm) with the immobilized HRP. The enzyme reactor was filled with 2.5 mmol/l boric acid and 50 mmol/l Tris-HCl buffer, pH 7.5. At 4°C, the reactor was stable for 6 months and served to measure more than 2000 samples.

2.4. Instrumentation

A schematic diagram of the assay system for urinary 1,5-AG is shown in Fig. 2. The color-developing solution and the carrier solution were delivered by two L-6000 double-plunger pumps (Hitachi, Tokyo, Japan) at flow-rates of 1 ml/min. A 2-μl sample was injected into the carrier stream by an AS-8010 sample injector (Tosoh) equipped with a 2-μl sample loop every 7 min. The sample was first introduced into the separation column. After passage through the column, the sample was mixed with the color-developing solution and then introduced into the immobilized enzyme reactor. The resulting colored product was detected in an SPD-10AV spectrophotometer (Shimadzu, Kyoto) at 727 nm. The peak area was recorded with a D-2500 Chromato-Integrator (Hitachi). The separation column and the immobilized enzyme reactor were kept at 25°C in an L-5030 column oven (Hitachi).

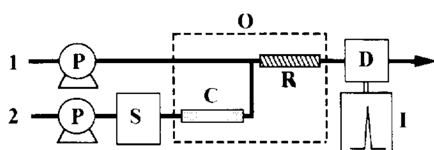

Fig. 2. Schematic diagram of the flow system: P, pump; S, sample injector with a 2-μl loop; D, spectrophotometer; I, chromato-integrator; O, column oven; C, separation column; R, immobilized enzyme reactor; 1, color-developing solution; 2, carrier solution.

2.5. Solutions

The carrier solution was 5 mmol/l boric acid in distilled water and the color-developing solution was 7.5 μmol/l of DA-64 in 0.1 mol/l Tris-HCl buffer, pH 7.5, containing 0.4 g/l of Triton X-100. They were stored at room temperature until use. 1,5-AG standard solutions were prepared in distilled water and stored at 4°C.

2.6. Refractive index detection

A refractive index detector (Shodex RI SE-61, Showadenko, Tokyo) was used to determine retention times for substances that reacted with PROD instead of the spectrophotometer in the system. The color-developing solution was replaced with distilled water. Authentic substances (50 mmol/l) were used for the analysis.

2.7. Collection and storage of samples

Urine samples were provided by the diabetic section of a general hospital and healthy volunteers. They were stored at −20°C until the analysis.

3. Results

We measured many urine samples from patients with diabetes mellitus and healthy subjects to confirm the specificity of our system. Peaks detected in the samples from diabetic patients were not different from those detected in the samples from healthy subjects. Typical chromatograms are shown in Fig. 3A,B. The peak corresponding to 1,5-AG was ob-

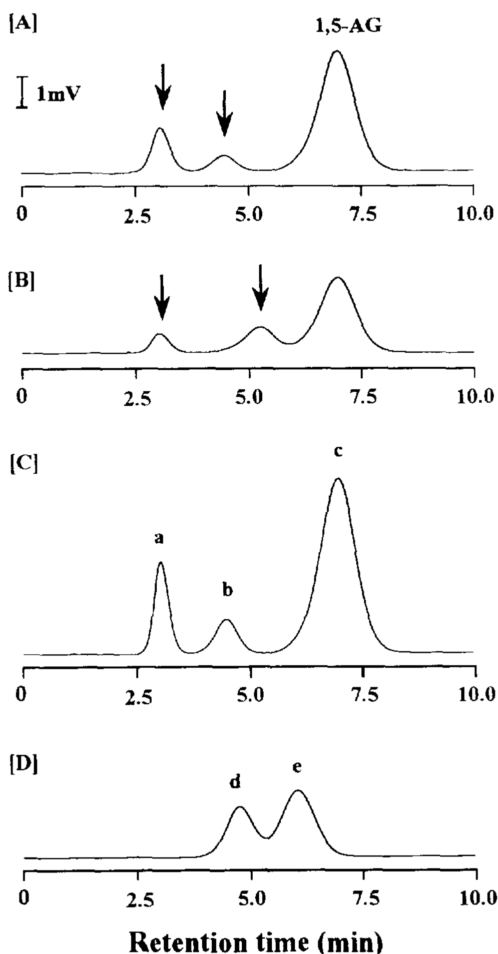

Fig. 3. Chromatograms of urine samples and authentic compounds. Chromatograms A and B were obtained from urine samples and the arrows in them show peaks of unknown substances. Chromatograms C and D were obtained from mixtures of authentic compounds: (a) levoglucosan (300 μmol/l); (b) *scyllo*-inositol (300 μmol/l); (c) 1,5-AG (50 μmol/l); (d) methyl-α-D-glucopyranoside (3 mmol/l); (e) methyl-β-D-glucopyranoside (300 μmol/l).

tained at a retention time of 7.0 min and the unknown peaks were observed in front of the 1,5-AG peak. The peak at the retention time of 3.1 min was found in both chromatograms. The peaks at 4.5 and 5.3 min were found in the chromatograms A and B, respectively. However, these three peaks were well separated from the 1,5-AG peak. No other peak was observed, even when monitoring was prolonged for 2 h after the elution of 1,5-AG. All the peaks appeared 2 min after the injection and disappeared within 9 min.

To identify the substances detected in our system, we examined the substrate specificity of the immobilized enzyme reactor and the performance of the separation column (Table 1). First we tested the system without the separation column. The immobilized PROD showed high relative activity with 1,5-AG, D-glucose, D-allose, D-galactose, L-idose, L-sorbose, D-xylose, D-glucono-δ-lactone; in contrast, with other D-forms of hexoses and pentoses PROD exhibited relative activities of less than 3% of 1,5-AG, and with linear alditols it did not show reactivity (data not shown). The reactivity of PROD with methyl-α-D-glucopyranoside, methyl-β-D-glucopyranoside and levoglucosan (1,6-anhydroglucopyranose) was slight and it was the same as with *myo*-inositol and *scyllo*-inositol.

Then the substances that reacted with PROD were tested by the system with the separation column. Sugars, including D-forms of hexoses and pentoses not shown in Table 1 but which exhibited slight reactivities were not detected when the tests were performed at the concentration of 150 μmol/l. D-Glucono-δ-lactone and *myo*-inositol were also not detected. 1,5-AG, methyl-α-D-glucopyranoside, methyl-β-D-glucopyranoside, levoglucosan and *scyllo*-inositol were detected. The retention times for levoglucosan, *scyllo*-inositol, methyl-α-D-glucopyranoside and methyl-β-D-glucopyranoside were 3.0, 4.5, 4.7 and 6.0 min, respectively (Fig. 3C,D).

Finally, we tested the ability of the separation column to trap the substrates by injecting high concentrations of them. Glucose, the main interfering substance found in urine, was completely trapped in the column when injected at concentrations of up to 500 mmol/l. Other sugars tested, D-glucono-δ-lactone and *myo*-inositol were trapped at concentrations of up to 500, 500 and 50 mmol/l, respectively (Table 1).

We also investigated, using the refractive index detector, whether or not the substrates were eluted from the separation column. 1,5-AG, levoglucosan, *scyllo*-inositol, methyl-α-D-glucopyranoside and methyl-β-D-glucopyranoside were detected at retention times equal to those determined by the colorimetric analysis with the immobilized enzyme reactor. *Myo*-inositol was not detected.

Table 1
Specificity of the system

Substrate	Relative activity[a] (%)		Concentration undetectable with separation column[b] (mmol/l)
	Without separation column	With separation column	
1,5-AG	100	102	–[c]
D-Glucose	124	0	500
D-Allose	58	0	–
D-Galactose	57	0	500
L-Idose	35	0	–
L-Sorbose	64	0	500
D-Xylose	92	0	500
D-Glucono-δ-lactone	43	0	500
Methyl-α-D-glucopyranoside	0.5	0.4	–
Methyl-β-D-glucopyranoside	5.9	5.5	–
Levoglucosan	3.3	3.2	–
myo-Inositol	0.4	0	50
scyllo-Inositol	2.6	2.2	–

[a] Percentage of the peak area. 1,5-AG, sugars and glucono-δ-lactone were tested at the concentration of 150 μmol/l, while the others were tested at the concentration of 3 mmol/l and their peak areas at the concentration of 150 μmol/l were calculated.
[b] The highest concentrations tested; at these concentrations no signal was detected when the study was performed with the separation column.
[c] Not tested.

3.1. Calibration curve and sensitivity

We measured 1,5-AG in serially diluted standard solutions (1.2–300 μmol/l) to determine the linear range of the calibration curve. The linear range of the calibration curve extended up to 300 μmol/l. The linear regression equation was $y=6.65x-0.50$ mV s ($n=9$, $r=1.000$, $S_{y/x}=8.48$), where y is the peak area and x is the 1,5-AG concentration (μmol/l).

The detection limit was 1.2 μmol/l, for which the signal-to-noise ratio was 3.

3.2. Precision and capacity of the separation column

To assess the within-run precision of the system, three different urine samples were injected 10 times each. To evaluate the day-to-day precision and the capacity of the separation column, the same three samples and a urine sample containing 50 mmol/l of glucose, which was prepared by adding authentic glucose, were used. A freshly prepared separation column was used in these experiments. First, the three urine samples were assayed 10 times each and then the sample containing 50 mmol/l of glucose was injected 40 times. This cycle was repeated seven times (the sample containing glucose was not injected the last time) over 7 days. The first determination value for 1,5-AG in each sample and each cycle was taken to evaluate the day-to-day precision. In total, these samples were injected 450 times into the column. The separation column and the immobilized enzyme reactor were kept in the system during this test. The coefficient of variance was less than 3% (Table 2) and one separation column could be used for more than 450 injections of samples.

3.3. Analytical recovery

We performed analytical recovery tests at three concentrations of 1,5-AG in three urine samples. The recovery rate was 97–103% (mean 100%), as shown in Table 3.

3.4. Comparison with the GC–MS method

We compared the results obtained with our system and those obtained with the GC–MS method de-

Table 2
Precision of the assay for urinary 1,5-AG

Sample	Within-run[a]			Day-to-day[b]		
	Mean (μmol/l)	S.D. (μmol/l)	C.V. (%)	Mean (μmol/l)	S.D. (μmol/l)	C.V. (%)
A	22.6	0.53	2.3	22.5	0.61	2.7
B	66.2	0.86	1.4	65.9	0.84	1.3
C	159.0	1.90	1.2	157.5	2.41	1.5

[a] Number of replicates, 10.
[b] Number of replicates, 7.

Table 3
Analytical recovery of 1,5-AG added to urine

Sample	Initial 1,5-AG concentration in urine (μmol/l)	Increase in 1,5-AG concentration (μmol/l)		
		30	60	120
A	22.2	29.8 (99)[a]	60.6 (101)	121.1 (101)
B	65.6	30.0 (100)	61.8 (103)	122.2 (102)
C	155.1	29.2 (97)	59.5 (99)	117.6 (98)

[a] One volume of distilled water or 1,5-AG standard solutions at three concentrations was added to nine volumes of each of three urine samples. The recovery amounts of added 1,5-AG were calculated as the difference between the measured concentrations of the sample with added 1,5-AG standard solutions and that with added distilled water. Figures in parentheses are the percentages of recovery.

scribed by Kametani et al. [6] in 42 urine samples from healthy subjects and diabetic patients. The correlation is shown in Fig. 4.

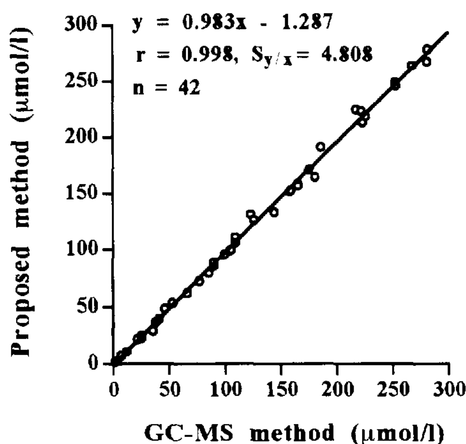

Fig. 4. Correlation between urinary 1,5-AG values determined by the GC–MS method and those determined using our system.

4. Discussion

4.1. Specificity of PROD

Unknown peaks were detected in front of the 1,5-AG peak even though a column packed with a H^+ form of a strongly cation-exchange resin, AG 50W-X8 (Bio-Rad Labs., Richmond, CA, USA), was placed between the separation column and the mixing point of the carrier solution and the color-developing solution. When the immobilized PROD resin was removed from the system, these peaks were not observed. This indicates that there are non-ionic substances that PROD reacts with in urine.

Janssen et al. [17] concluded that PROD reacted with substances which have a pyranoid ring with equatorial hydroxyl groups at C-2, C-3 and C-4 positions for aldose (corresponding positions for ketose and glucono-δ-lactone) in the chair form. They also said that the substituents at C-1 affected the reactivity (methyl-β-D-glucopyranoside and levoglucosan did not react with PROD).

According to our results, the immobilized enzyme reactor oxidized not only the substances which have the structure pointed out by Janssen et al., i.e. 1,5-AG, D-glucose, L-idose, D-xylose, L-sorbose and glucono-δ-lactone, but also others such as D-galactose, D-allose, methyl-β-D-glucopyranoside and levoglucosan. D-galactose and D-allose showed high reactivities. Undoubtedly, D-galactose and D-allose react with PROD. Levoglucosan, *scyllo*-inositol, methyl-α-D-glucopyranoside and methyl-β-D-glucopyranoside were injected into the separation column and detected in the eluate at intrinsic retention times which were the same times determined by the refractive index detector. We conclude that these substances are the substrates for the immobilized PROD in spite of their low reactivities. The reactivity of PROD with *myo*-inositol was demonstrated in the previous papers [11–13]. *Myo*-inostol and *scyllo*-inositol have a 6-membered carbon ring and equatorial hydroxyl groups in the chair form. As their structures resemble pyranose, it is likely that they react with PROD. In our system PROD showed a broader substrate specificity than that described by Janssen et al. The difference between our results and those of Janssen et al. is probably due to different assay conditions. In our system the reaction was done at lower concentrations of the substrate with a large amount of PROD in the reactor column.

4.2. Separation column

The separation column did not trap levoglucosan, *scyllo*-inositol, methyl-α-D-glucopyranoside and methyl-β-D-glucopyranoside. It is known that borate ions react easily with adjacent *cis*-hydroxyl groups to form negatively charged complexes, which interact with anion-exchange resins. Thus, *myo*-inositol, which has adjacent *cis*-hydroxyl groups, was trapped in the separation column, but *scyllo*-inositol which does not have them was not trapped. However, β-D-glucose, a major component of D-glucose in an aqueous solution, does not have adjacent *cis*-hydroxyl groups, but was trapped in the separation column. It is known that hydroxyl groups at the C-4 and C-6 positions of pyranose can also form complexes with borate ions. Furthermore, mutarotation and franose–pyranose interconversion are important steps in the formation of sugar-borate complexes [16]. In an aqueous solution, glucono-δ-lactone changed into D-gluconic acid and glucono-γ-lactone, and was trapped in the separation column. 1,5-AG, levoglucosan, *scyllo*-inositol and methyl-D-glucopyranosides which can not change their form were not trapped in the column, and some of them (1,5-AG and methyl-D-glucopyranosides) have hydroxyl groups at the C-4 and C-6 positions. We think that β-D-glucose was trapped through the mechanisms of mutarotation and franose–pyranose interconversion in the separation column. The borate complexes formed with compounds having hydroxyl groups at the C-4 and C-6 positions would interact weakly with the anion-exchange resin, because methyl-α-D-glucopyranoside, methyl-β-D-glucopyranoside and 1,5-AG were eluted after levoglucosan and *scyllo*-inositol.

When a high concentration of boric acid (250 mmol/l) was used as the carrier solution, glucose was eluted from the column about 1 h after the injection. However, we could inject urine samples 450 times into a fresh separation column at intervals of 7 min. The concentration of boric acid affects the affinity of a sugar-borate complex for an anion-exchange resin. We think that our success is due to using a very low concentration of boric acid (5 mmol/l) as the carrier solution.

We could not identify the substances detected in urine. Levoglucosan and *scyllo*-inositol, which showed similar retention times to those of the unknown peaks, exist in human urine [18,3] and they were probably detected as parts of unknown peaks in this study.

4.3. Assay performance

Although three unknown peaks were detected, they were well separated from the 1,5-AG peak and did not affect the quantification of urinary 1,5-AG. As all peaks were detected within 7 min from the beginning of the first peak, we set the injection interval at 7 min. Glucose, which is the main interfering substance in urine, was trapped in the separation column at a concentration of up to 500 mmol/l, so we think urinary glucose will not affect the determination of 1,5-AG in samples from diabetic patients. The analytical recovery and the correlation with GC–MS were satisfactory. Our system

does not need any pretreatment of a urine sample and can deal with many samples in a short time by automated successive injections every 7 min. This system can be used conveniently in routine work and is a useful tool in the determination of urinary 1,5-AG.

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