Quantification of Serum 1,5-Anhydroglucitol in Uremic and Diabetic Patients by Liquid Chromatography/Mass Spectrometry

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We developed a new method for measuring serum 1,5-anhydroglucitol (1,5-AG), using liquid chromatography/atmospheric pressure chemical-ionization mass spectrometry (LC/MS). With this method we measured serum 1,5-AG concentrations in uremic and diabetic patients and compared these values with those determined by an enzymatic method. Serum 1,5-AG concentrations were significantly less in the undialyzed and dialyzed uremic patients and in diabetic patients; the values in the dialyzed patients decreased after hemodialysis. In uremic patients with high concentrations of serum myo-inositol, the 1,5-AG values determined by LC/MS were significantly lower than those determined enzymatically. There was no significant correlation between 1,5-AG and fructosamine in the uremic patients. These results demonstrate that serum 1,5-AG cannot be used as an index for glycemic control in uremic patients and that the LC/MS method is indicated in uremic patients with high concentrations of myo-inositol.

Indexing Terms: myo-inositol/hemodialysis

1,5-Anhydro-D-glucitol (1,5-AG), also called 1-deoxyglucose, is a 1-deoxy form of glucopyranose and is one of the main polyols in human serum and cerebrospinal fluid. 1,5-AG was first detected in human cerebrospinal fluid by Pitkänen, using gas chromatography/mass spectrometry (GC/MS) (1). 1,5-AG has been detected in the serum of uremic patients (2). Because serum concentrations are decreased in diabetic patients (3–11), serum 1,5-AG has been used as a marker of glycemic control in diabetics (12) and as a screening criterion for diabetes mellitus (13).

The quantification of 1,5-AG in serum or plasma has been performed by GC or GC/MS (2–12). However, these methods require derivatization and laborious sample preparation steps, and cannot be used routinely for the clinical examination of 1,5-AG. A simple enzymatic method for measuring 1,5-AG was developed, based on the oxidation of 1,5-AG by pyranose oxidase, which requires plasma preparation with minicolumns to remove other interfering sugars and sugar alcohols (14). However, the enzymatic method also recognizes myo-inositol, which cannot be completely removed by the minicolumn (14). In uremic patients, serum concentrations of myo-inositol are markedly increased (2, 15), leading to overestimation of serum 1,5-AG concentrations.

We developed a new method for the quantification of serum 1,5-AG, using liquid chromatography/atmospheric pressure chemical-ionization mass spectrometry (LC/APCI-MS), which does not require derivatization. With this method, we measured serum 1,5-AG concentrations in uremic and diabetic patients to determine whether 1,5-AG could serve as an index for glycemic control and compared the values with those determined by the enzymatic method.

Materials and Methods

1,5-AG and d6-1,5-AG were gifts from Nippon-Kayaku Co. (Tokyo, Japan). Myo-inositol was obtained from Yoneyama Chemical Industries (Osaka, Japan). All other chemicals used were of analytical grade.

Subjects

The study included 25 diabetic patients (16 men and 9 women, mean ± SD ages 57.4 ± 11.5 years, range 35–80), 20 nondiabetic uremic undialyzed patients (15 men and 5 women, ages 53.9 ± 13.2 years, 22–71), 22 nondiabetic dialyzed patients (12 men and 10 women, ages 56.3 ± 12.4 years, 35–74), and 8 diabetic dialyzed patients (7 men and 1 woman, ages 58.4 ± 10.1 years, 39–68). Serum samples were taken before and after hemodialysis (HD). The controls were 20 healthy subjects (10 men and 10 women, ages 46.1 ± 19.1 years, 23–76). The samples were obtained from these subjects in accordance with the Helsinki Declaration of 1975, as revised in 1983.

Mean ± SD serum concentrations (and ranges) of creatinine were 0.088 ± 0.018 (0.071–0.159) mmol/L in diabetic patients, 0.51 ± 0.044 (0.19–1.73) mmol/L in uremic undialyzed patients, 1.18 ± 0.25 (0.69–1.69) mmol/L in nondiabetic dialyzed patients, 1.06 ± 0.27 (0.57–1.39) mmol/L in diabetic dialyzed patients, and 0.088 ± 0.018 (0.071–0.106) mmol/L in healthy subjects.

Sample Preparation

After addition of 147 nmol of d6-1,5-AG as an internal standard, 0.5 mL of serum was deproteinized with 1 mL of ethanol. After centrifugation for 10 min at 1000g, the supernatant was concentrated to 0.5 mL under a nitrogen stream. The solution was applied to a Bond Elut
SCX cartridge (cation-exchange, 100 mg/mL; Analytichem International, Harbor City, CA) and eluted with 2 mL of distilled water. The collected eluate was then applied to a Bond Elut SAX cartridge (anion-exchange, 100 mg/mL; Analytichem International) and eluted with 2 mL of distilled water. The eluate was freeze-dried and the residue was dissolved in 100 μL of methanol/distilled water (1:1 by vol). We injected 20 μL of the sample into the LC/APCI-MS analyzer.

LC/APCI-MS

We used a Hitachi M-1000S quadrupole mass spectrometer equipped with APCI and a Hitachi 6200 LC pump and a Gelpack GL-C84Z column (150 × 6 mm (i.d.), 10-μm particles; Hitachi-Kasei, Tokyo, Japan) containing sulfonated styrene-divinylbenzene copolymer with zine ions for LC/MS. Acetonitrile/distilled water (8:2 by vol) was eluted at 1.0 mL/min. The column temperature was 80°C. The separation of polysols was better at 80°C than at room temperature, and the column was stable at 80°C. To accelerate ionization, we added 10 mL/L chloroform in methanol at 0.5 mL/min to the eluate after it had passed through the HPLC column.

Mass spectra were recorded with negative-ion APCI (NI-APCI). The drift voltage was −25 V, vaporizer temperature 250°C, and desolvator temperature 399°C.

To quantify 1,5-AG and myo-inositol in the serum samples, we prepared calibration curves with selected-ion monitoring (SIM) chromatograms. We added 1,5-AG and myo-inositol, 1.52 to 152 nmol, to 0.5 mL of distilled water. After addition of 147 nmol of d₆-1,5-AG as an internal standard, these solutions were processed as described above and analyzed by LC/APCI-MS. Calibration curves relating the concentrations of 1,5-AG and myo-inositol to their peak-area ratios at m/z 199 (1,5-AG) and m/z 215 (myo-inositol) [representing their (M + Cl)⁻ ions], and to the internal standard at m/z 205, were obtained from the SIM chromatograms.

Enzymatic Method for Quantification of Serum 1,5-AG

We also measured serum 1,5-AG by an enzymatic method, using a kit (Nippon-Kayaku) (14). Briefly, plasma samples deproteinized with trichloroacetic acid were passed through a two-layer minicolumn packed with strongly basic anion- (upper layer) and strongly acidic cation- (lower layer) exchange resins. 1,5-AG was efficiently recovered in the flow-through fraction. Hydrogen peroxide formed in the enzymatic oxidation of 1,5-AG by pyranose oxidase was detected by a standard method with an enzymatic color-developing system.

Serum Fructosamine

We measured serum fructosamine by using the fructosamine test Roesch II (Japan Rosch, Tokyo, Japan).

Statistical Analysis

All results are expressed as mean ± SE. Statistical analysis was by analysis of variance for multiple comparisons and by the paired t-test for paired data.

Results

The correlation coefficient of the calibration line relating the concentration of 1,5-AG (x) to its peak area ratio (y) was 0.9999 (y = 0.0194 + 0.00584x). The standard deviations of the intercept and the slope were 0.0083 and 0.000047, respectively, and the standard error of the estimates was 0.0064. For myo-inositol, r = 0.9996 (y = 0.0178 + 0.00406x); the standard deviations of the intercept and the slope were 0.020 and 0.00012, respectively, and the standard error of the estimates was 0.015. Intra- and interassay CVs for 1,5-AG were 6.1% (n = 5) and 7.2% (n = 5) at 160 μmol/L, respectively, and 8.8% (n = 5) and 9.2% (n = 5) at 13 μmol/L, respectively. Intra- and interassay CVs for myo-inositol were 8.7% (n = 5) and 9.8% (n = 5) at 220 μmol/L, respectively, and 10.2% (n = 5) and 10.8% (n = 5) at 17 μmol/L, respectively. The intra- and interassay CVs were determined in serum to which 1,5-AG and myo-inositol had been added. The limit of quantification for serum 1,5-AG was 1 μmol/L.

Figure 1 shows the NI-APCI mass spectra of 1,5-AG, d₆-1,5-AG, and myo-inositol. With postcolumn addition of 10 mL/L chloroform in methanol to accelerate ionization, the (M + Cl)⁻ ions were detected as base peaks in the mass spectra. By comparing (M + Cl)⁻ ions with (M − H)⁻ ions, one can easily determine their molecular masses. Fig. 2 shows the SIM chromatograms of standards, normal serum, diabetic serum, uremic undialyzed serum, uremic pre-HD serum, and uremic post-HD serum. The mass spectra and the retention times of 1,5-AG, d₆-1,5-AG, and myo-inositol were identical to those of the authentic compounds.

Table 1 shows the serum concentrations of 1,5-AG, myo-inositol, and fructosamine in diabetic, uremic undialyzed, and dialyzed patients. Serum 1,5-AG concen-
trations were significantly decreased in all patients. These values decreased significantly after HD; the mean reduction rate was 54.0%. 1,5-AG concentrations were not significantly different in nondiabetic dialyzed and diabetic post-HD patients, although serum fructosamine concentrations were significantly (P < 0.01) higher in the diabetic dialyzed patients than in the nondiabetic patients after HD. Mean serum 1,5-AG concentrations determined by LC/MS were significantly lower in the uremic undialyzed and dialyzed patients than those determined by the enzymatic method. However, there were no significant differences between the two methods in serum 1,5-AG measurements of normal subjects and diabetic patients.

The amounts of serum myo-inositol were markedly increased in the undialyzed and dialyzed uremic patients but not in the diabetics. Myo-inositol concentrations decreased significantly (P < 0.01) after HD; the mean reduction rate was 63%.

Serum fructosamine concentrations were significantly increased not only in the diabetic undialyzed and diabetic dialyzed patients but also in the nondiabetic dialyzed patients. Serum fructosamine showed a negative correlation with serum 1,5-AG (r = −0.52, P < 0.01) in diabetic patients. However, there were no significant correlations between fructosamine and 1,5-AG in uremic undialyzed and dialyzed patients, indicating that serum 1,5-AG cannot be used as a

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**Table 1. Serum concentrations (μmol/L) of 1,5-AG, myo-inositol, and fructosamine in diabetic, uremic undialyzed, and healthy subjects.**

<table>
<thead>
<tr>
<th></th>
<th>LC/APCI-MS</th>
<th>Enzymatic</th>
<th>myo-inositol</th>
<th>Fructosamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>151.8 ± 11.6</td>
<td>159.8 ± 9.8</td>
<td>37 ± 7</td>
<td>261 ± 5</td>
</tr>
<tr>
<td>Diabetic</td>
<td>50.0 ± 8.5</td>
<td>56.7 ± 8.5</td>
<td>52 ± 5</td>
<td>429 ± 29</td>
</tr>
<tr>
<td>Uremic undialyzed</td>
<td>85.4 ± 15.2</td>
<td>106 ± 21.3</td>
<td>244 ± 53</td>
<td>282 ± 13</td>
</tr>
<tr>
<td>Dialyzed diabetic (pre-HD)</td>
<td>15.2 ± 3.0</td>
<td>23.2 ± 6.7</td>
<td>311 ± 37</td>
<td>439 ± 26</td>
</tr>
<tr>
<td>(post-HD)</td>
<td>7.9 ± 1.8</td>
<td>15.2 ± 1.2</td>
<td>121 ± 15</td>
<td>477 ± 36</td>
</tr>
<tr>
<td>Dialyzed nondiabetic (pre-HD)</td>
<td>7.9 ± 1.2</td>
<td>28.0 ± 1.8</td>
<td>270 ± 46</td>
<td>328 ± 9</td>
</tr>
<tr>
<td>(post-HD)</td>
<td>7.9 ± 1.2</td>
<td>14.6 ± 0.6</td>
<td>78 ± 15</td>
<td>332 ± 14</td>
</tr>
<tr>
<td>ANOVA</td>
<td><em>P &lt; 0.05</em></td>
<td><em>P &lt; 0.01</em></td>
<td><em>P &lt; 0.05</em></td>
<td><em>P &lt; 0.01</em></td>
</tr>
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</table>

*P < 0.05, *P < 0.01 as compared with normal subjects by ANOVA; †P < 0.05, ‡P < 0.01 as compared with enzymatic method by paired t-test.

Normal ranges for 1,5-AG (77–270 μmol/L by LC/MS and 81–287 μmol/L by enzymatic method) were calculated as mean ± 2SD after logarithmic conversion.
marker for glycemic control in uremic patients, whether dialyzed or not.

In uremic undialyzed patients, serum 1,5-AG showed a negative correlation with serum creatinine \((r = -0.47, P < 0.05)\). There were good correlations in diabetic patients \((r = 0.94)\) (Fig. 3) and in normal subjects \((r = 0.91)\) between the LC/APCI-MS method \((x)\) and the enzymatic method \((y)\) for 1,5-AG measurements. The standard deviations of the intercept and the slope of the regression line in normal subjects \((y = 11.0 + 0.995x)\) were 18.6 and 0.133, respectively, and the standard error of the estimates was 3.54. However, results by the two methods were poorly correlated in the dialyzed patients \((r = 0.39)\) (Fig. 4).

Discussion

We developed a new method for measuring serum 1,5-AG by using LC/MS and demonstrated that serum 1,5-AG concentrations were reduced not only in diabetic patients but also in nondiabetic uremic patients. Although serum 1,5-AG has been used as an index for glycemic control in diabetic patients, it cannot be used as an index for glycemic control in uremic patients. Given the excellent agreement between the enzymatic method and the LC/MS method for measuring 1,5-AG in nonuremic diabetic patients with low concentrations of myo-inositol, the simple enzymatic method is suitable to monitor these patients. However, LC/MS is indicated in uremic patients with high concentrations of myo-inositol.

The newly developed LC/MS method is simpler and more rapid than GC/MS because it does not require derivatization. The LC/MS method is specific to 1,5-AG and does not recognize myo-inositol, which is markedly increased in uremic serum. Since the enzymatic method also reacts with myo-inositol \((14)\), serum 1,5-AG concentrations in uremic patients may be overestimated. We demonstrated that the serum 1,5-AG concentrations determined by the LC/MS method were significantly lower than those determined by the enzymatic method in the uremic undialyzed and dialyzed patients. The accumulation of myo-inositol, which interferes with the enzymatic method, can explain the poor correlation between the LC/MS method and the enzymatic method for the measurement of serum 1,5-AG in dialyzed patients.

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 \((7, 16)\). There is little diurnal variation, and the concentration is not influenced by the intake of food or the glucose load \((7, 9)\). The slow turnover of serum 1,5-AG observed in rat studies \((half-life, 120 \text{h})\) \((17)\) 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 \((16, 18)\). 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 \((16)\) 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.
That the reduction of serum 1,5-AG in dialyzed patients is mainly due to its removal by HD is evident from the significantly decreased concentrations after HD, with the mean reduction rate being 54.0%. However, it is not yet clear why serum 1,5-AG is reduced in the uremic undialyzed patients. End-stage renal disease, even when caused by glomerular sclerosis, is usually accompanied by renal tubular dysfunction. In fact, \( \beta_2 \)-microglobulin, normally reabsorbed by the renal tubules, is excreted in large quantities in urine in end-stage renal disease patients because of the impaired renal tubular reabsorption. Likewise, the impaired renal reabsorption of 1,5-AG may cause a urinary loss, leading to decreased serum 1,5-AG concentrations in uremic undialyzed patients.

Serum fructosamine concentrations were increased not only in diabetic patients and diabetic dialyzed patients, but also in nondiabetic dialyzed patients. This may be due to increased nonenzymatic glycosylation of proteins in terminal uremia. Nonenzymatically glycosylated hemoglobin and plasmatic glycosylated proteins were increased in end-stage renal disease patients (19). Normal fasting and high postprandial glucose values are also often observed in these patients.

In summary, serum 1,5-AG concentrations were reduced in undialyzed and dialyzed uremic patients, as demonstrated by a newly developed LC/MS method, and thus 1,5-AG cannot be used as a marker for glycemic control in the uremic patients. However, these results do not exclude the usefulness of 1,5-AG as a marker for glycemic control in diabetic patients and as a screening criterion for diabetes mellitus.

References