Gas Chromatographic–Mass Spectrometric Identification of Isosaccharino-1,4-lactone in Human Serum and Urine

Toshimitsu Niwa, Kenji Maeda, Toyokazu Ohki, Akira Saito, Jinsaku Sakakibara, and Kaizo Kobayashi

We have identified α-isosaccharino-1,4-lactone and β-isosaccharino-1,4-lactone in the ethyl acetate extract of an ultrafiltrate of blood and of normal human urine, using high-resolution gas chromatography–mass spectrometry. The ultrafiltrate of blood was obtained from three patients on hemodialysis, one with psoriasis vulgaris, one with uremia, and one with amyotrophic lateral sclerosis. The concentration of β-isosaccharino-1,4-lactone in the ultrafiltrate was two- to threefold that of α-isosaccharino-1,4-lactone. Mass fragmentography showed that isosaccharino-1,4-lactone is normally present in human serum in a concentration of 3.4 (SD 1.5) mg/L. In physiological fluid, isosaccharino-1,4-lactone apparently is present in its hydrated form, isosaccharinate.

High-resolution gas chromatography/mass spectrometry is becoming increasingly important in the clinical chemistry laboratory for separating and identifying components of physiological fluids (1, 2). While investigating the acidic fraction in ultrafiltrate of uronic serum (3, 4), we detected an unknown compound that was also detected in the acidic fraction of ultrafiltrate of serum from psoriasis patients. In the latter compound is one of the major components. We have identified it as isosaccharino-1,4-lactone and determined its concentration in normal human serum and its urinary excretion. These findings are reported here for the first time.

Materials and Methods

Chemicals

Trimethylsilylating agents, N,O-bis(trimethylsilyl)acetamide and trimethylchlorosilane, were purchased from Pierce Chemical Co., Rockford, IL 61105; α-lactose, maltose, calcium hydroxide, and oxalic acid from Yoneyama Chemical Industries Ltd., Osaka, Japan; p-(n-amyl)benzoic acid from Tokyo Kasei Kogyo Co., Tokyo, Japan; and pyridine-d$_6$, H$_2$O, and D$_2$HCl from E. Merck, Darmstadt, F.R.G.

α-D-Isosaccharino-1,4-lactone was synthesized according to the method of Whistler and BeMiller (5) and the structure of the purified product was confirmed by infrared spectroscopy and $^1$H-NMR and $^{13}$C-NMR spectroscopy. Because it is difficult to separate β-D-isosaccharino-1,4-lactone from α-D-isosaccharino-1,4-lactone, we synthesized a mixture of the two according to the method of Corbett and Kenner (6). The purified product was confirmed to be a mixture of the α- and β-isomers by $^{13}$C-NMR spectroscopy (Figure 1).

Samples and Sample Preparation

Blood and urine samples. Blood ultrafiltrate (1.5 L) was obtained from three patients on hemodialysis: one with psoriasis vulgaris, one with uremia, and one with amyotrophic lateral sclerosis. The hemodialysis was by the extracorporeal ultrafiltration method (7). Rhone Poulenc hemodialyzers, RP-6, were used. The psoriatic patient and the patient with amyotrophic lateral sclerosis had normal renal function.

Samples of serum were obtained from 10 healthy adults (seven men, three women) after overnight fasting; 24-h urine samples were collected from two healthy adults. The ultrafiltrate, the serum samples, and the urine samples were kept at −20°C until analysis.

Sample preparation. Two hundred milliliters of the ultrafiltrate was acidified to pH 1 with HCl, saturated with NaCl, and mixed with 800 mL of ethyl acetate. This mixture was shaken for 1 h. To determine only the relative retention times of the extracted compounds on gas chromatography, we added 40 μg of p-(n-amyl)benzoic acid to the extract.

The extract was dried over anhydrous Na$_2$SO$_4$, evaporated with a rotary evaporator, trimethylsilylated with 200 μL of N,O-bis(trimethylsilyl)acetamide and 50 μL of trimethylchlorosilane, washed with 1 mL of distilled water, extracted with hexane, and concentrated to 10 μL, 2 μL of which was subjected to gas chromatography on a glass capillary column and mass spectrometry.

Serum was filtered through a CF-25 membrane filter (Amicon Co., Lexington, MA 02173). As an internal standard, 0.5 μg of p-(n-amyl)benzoic acid in 50 μL of ethyl acetate was added to 1 mL of the serum ultrafiltrate, which was then acidified to pH 1 with HCl, saturated with NaCl, and extracted three times with 3-mL portions of ethyl acetate. The extract was dried over anhydrous Na$_2$SO$_4$, evaporated in a nitrogen stream, and trimethylsilylated with 40 μL of N,O-bis(trimethylsilyl)acetamide and 10 μL of trimethylchlorosilane at 70°C for 1 h. Three μL of the sample was subjected to combined gas chromatography (packed column)/mass spectrometry.

Fifty milliliters of a 24-h urine sample was acidified to pH 1, saturated with NaCl, and mixed with 150 mL of ethyl acetate. The mixture was shaken for 1 h. After 200 μg of p-(n-amyl)benzoic acid was added to the extract, it was dried, evaporated, and trimethylsilylated with 200 μL of N,O-bis(trimethylsilyl)acetamide and 50 μL of trimethylchlorosilane. After washing with 1 mL of distilled water, the derivatives were extracted with hexane and concentrated into 10 μL. Two microliters of the sample was subjected to gas chromatography on a glass capillary column and mass spectrometry.

Analytical Procedures

Thin-layer chromatography. The ethyl acetate extract from the normal human urine (40 mL) was subjected to thin-layer chromatography on silica gel 60 (E. Merck) with the solvent system chloroform/methanol (60/40, by vol); α-D-isosaccharino-1,4-lactone had an $R_f$ value of 0.81 in this
system. A narrow band corresponding to this $R_t$ value was scraped off and the substances were eluted with ethanol. After 150 $\mu$L of $p$-(n-amyl)benzoic acid was added to the eluate for use in determining the relative retention times of the substances, the eluate was evaporated and trimethylsilylated with 30 $\mu$L of $N,O$-bis(trimethylsilyl)acetamide and 10 $\mu$L of trimethylchlorosilane. Two microliters of the sample was subjected to gas chromatography on a splitless glass capillary column, followed by mass spectrometry.

Gas chromatography-mass spectrometry. We used a gas chromatograph (JGC-20K; JEOL Ltd., Tokyo, Japan) combined with a mass spectrometer (JMS D-300; JEOL Ltd.). For identification, the gas chromatograph was equipped with an OV1 open tubular glass capillary column (30 m $\times$ 0.25 mm). The carrier gas was helium, with a flow rate of 0.64 mL/min. The split ratio was 30:1. The column temperature was programmed from 100 $^\circ$C to 250 $^\circ$C at 3 $^\circ$C/min. Electron-impact ionization mass spectra were recorded at an ionizing energy of 22 eV, an ionization current of 300 $\mu$A, a separator temperature of 270 $^\circ$C, an ion source temperature of 210 $^\circ$C, and an accelerating voltage of 3 kV. Chemical ionization mass spectra were recorded with methane as the reactant gas. Ionizing energy was 260 eV. The other conditions were the same for electron impact ionization.

For high-resolution mass spectrometry we included a data-processing system (JMA-2000; JEOL Ltd.). Ionizing energy was 70 eV. Ionization current was 300 $\mu$A. Accelerating voltage was 3 kV. Peak matching measurements were performed, with perfluorokerosene as standard.

The sample prepared by use of thin-layer chromatography was examined by splitless glass capillary column gas chromatography/mass spectrometry. The gas chromatograph 5710A (Hewlett-Packard Co., Palo Alto, CA 94303) was equipped with a 30-m OV101 open tubular glass capillary column, and directly coupled to the mass spectrometer. The carrier gas was helium, with a flow rate of 0.67 mL/min. The temperature was programmed from 100 $^\circ$C/min to 260 $^\circ$C/min, at 3 $^\circ$C/min. Electron-impact ionization mass spectra were recorded at an ionizing energy of 70 eV, an ionization current of 300 $\mu$A, an interface temperature of 240 $^\circ$C, an ion source temperature of 210 $^\circ$C, and an accelerating voltage of 3 kV.

For quantitation, a mass-fragmentographic technique was used. The gas chromatograph was equipped with 1% OV101 (2 m $\times$ 2 mm) on Gas Chrom Q (80–100 mesh). The carrier gas was helium with a flow rate of 30 mL/min. The column temperature was programmed from 200 $^\circ$C to 250 $^\circ$C at 4 $^\circ$C/min. Two ions, 348 and 249, were monitored by the multiple ion detection unit. The former, $(M - 30)^+$, was used to quantitate isosaccharino-1,4-lactone. The latter, $(M - 15)^+$, was monitored for $p$-(n-amyl)benzoic acid.

Quantitation of isosaccharino-1,4-lactone in human serum. Because we could not separate $\alpha$- and $\beta$-isosaccharino-1,4-lactone on a 1% OV101 packed column (2 m $\times$ 2 mm), we obtained a standard curve with the packed column by using standard solutions with concentrations ranging from 0.3 to 10 $\mu$g of $\alpha$-D-isosaccharino-1,4-lactone per milliliter of distilled water. After 0.5 $\mu$L of $p$-(n-amyl)benzoic acid in 50 $\mu$L of ethyl acetate was added, these solutions were acidified, saturated with NaCl, and extracted three times with 3-mL portions of ethyl acetate. The extract was dried, evaporated, and trimethylsilylated. A standard curve relating the concentrations of $\alpha$-D-isosaccharino-1,4-lactone to the ratios of peak heights of $\alpha$-D-isosaccharino-1,4-lactone (m/z 348) to $p$-(n-amyl)benzoic acid (m/z 249) was obtained from the mass frag-
mentogram. The standard curve was linear over the entire range.

Recovery and Reproducibility throughout the Entire Procedure

To assess the analytical recovery for the whole procedure, we added 10 μg of α-d-isosaccharino-1,4-lactone in 0.5 mL of distilled water to 0.5 mL aliquots of ultrafiltrate from the psoriatic patient. After 0.5 μg of p-(n-amyl)benzoic acid was added, these solutions were acidified, saturated with NaCl, and extracted three times with 3 mL portions of ethyl acetate. The extract was dried, evaporated, and trimethylsilylated. The amount of α-D-isosaccharino-1,4-lactone found in the solutions was determined from the peak height ratio and the standard curve. As shown in Table 1, the calculated mean recovery of added α-D-isosaccharino-1,4-lactone was 94 (SD 9).

One-milliliter aliquots of distilled water to which 1 μg of α-D-isosaccharino-1,4-lactone was added were analyzed. The peak-height ratio was 0.056 (SD 0.0069, n = 5, CV 12.3%).

Nuclear Magnetic Resonance Spectroscopy (NMR)

3H-NMR spectra were recorded with a JNM MH-100 spectrometer (JEOL Ltd.). 13C-NMR spectra were recorded with a JNM FX-100 spectrometer (JEOL Ltd.). The samples were dissolved in pyridine-d₅ or in 2H₂O. Tetramethylsilane was used as an internal or external reference.

Results

Identification of Isosaccharino-1,4-lactone in Blood Ultrafiltrate

Figure 2 shows the total ion monitoring chromatogram of the trimethylsilylated ethyl acetate extract from the psoriatic ultrafiltrate with an open tubular glass capillary column. Peaks 1 and 2 show the same mass spectra as shown in Figures 3 and 4, respectively. This suggests that the peak 1 compound and the peak 2 compound are diastereomers. Chemical ionization mass spectra of the peaks indicate molecular ions at m/z 378. High-resolution data are summarized in Table 2. The composition of the molecular ion indicates a tri-(trimethylsilyl) derivative of original composition C₆H₁₀O₅. The peak 1 compound and the trimethylsilylated derivative of α-D-isosaccharino-1,4-lactone had identical retention times in the OV1 open tubular glass capillary column (Figure 2) and identical mass spectra (Figure 3). The compound represented by peak 2 and the trimethylsilylated β-D-isosaccharino-1,4-lactone had identical retention times (Figure 2) and identical mass spectra (Figure 4). Isosaccharino-1,4-lactone was also detected in the uremic ultrafiltrate and in the ultrafiltrate from a patient with amyotrophic lateral sclerosis. It is yet unknown whether the isosaccharino-1,4-lactone is in the D- or L- form. The concentration of β-isosaccharino-1,4-lac-

<table>
<thead>
<tr>
<th>m/z</th>
<th>Observed m/z</th>
<th>Error (milli mass)</th>
<th>Unsaturation</th>
<th>Probable composition</th>
<th>Fragmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>378</td>
<td>378.1743</td>
<td>3.0</td>
<td>2.0</td>
<td>C₁₅H₂₂O₅Si₃</td>
<td>M⁺</td>
</tr>
<tr>
<td>363</td>
<td>363.1475</td>
<td>0.0</td>
<td>2.5</td>
<td>C₁₄H₂₁O₅Si₃</td>
<td>(M - CH₃)⁺</td>
</tr>
<tr>
<td>348</td>
<td>348.1599</td>
<td>-0.5</td>
<td>2.0</td>
<td>C₁₄H₂₂O₅Si₃</td>
<td>(M - CH₂O)⁺</td>
</tr>
<tr>
<td>245</td>
<td>245.1017</td>
<td>-1.0</td>
<td>2.5</td>
<td>C₁₀H₂₁O₃Si₂</td>
<td>(M - CH₂O - CH₂OTMS)⁺</td>
</tr>
<tr>
<td>231</td>
<td>231.1210</td>
<td>2.3</td>
<td>1.5</td>
<td>C₁₀H₂₂O₃Si₂</td>
<td>(M - CH₂O - COOTMS)⁺</td>
</tr>
<tr>
<td>217</td>
<td>217.0773</td>
<td>5.7</td>
<td>2.5</td>
<td>C₉H₁₇O₃Si₂</td>
<td></td>
</tr>
<tr>
<td>203</td>
<td>203.0949</td>
<td>2.6</td>
<td>1.5</td>
<td>C₉H₁₈O₂Si₂</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. High-Resolution Mass Spectral Data
Fig. 3. Electron impact ionization mass spectrum of trimethylsilylated α-β-isosaccharino-1,4-lactone (upper spectrum) and electron impact ionization mass spectrum of peak 1 compound (lower spectrum) in the psoriatic chromatogram of Fig. 2.

These spectra were recorded under the following conditions: ionizing energy 22 eV, ionization current 300 μA, separator temperature 270 °C, ion source temperature 210 °C, and accelerating voltage 3kV.

Fig. 4. Electron impact ionization mass spectrum of trimethylsilylated β-α-isosaccharino-1,4-lactone (upper spectrum) and electron impact ionization mass spectrum of peak 2 compound (lower spectrum) in the psoriatic chromatogram of Fig. 2.

Spectra recorded under the same conditions as in Fig. 3.
$lsosaccharino-1,4-lactone$, extract showed mass spectrometry. The Fig. peaks methylsilylated spectra, normal identifications: 1, 3-methyladipic acid; 2, $p$-hydroxybenzoic acid; 3, $p$-hydroxyphenylacetic acid; 4, suberic acid; 5, $\alpha$-isosaccharino-1,4-lactone; 6, $\beta$-isosaccharino-1,4-lactone; 7, $p$-(n-amyl)benzoic acid (internal standard), and 8, homovanillic acid

![Graph](https://i.imgur.com/3G5Q5.png)

Fig. 5. Total ion monitoring chromatogram of the trimethylsilylated ethyl acetate extract from normal human urine

Peak identifications are: 1, 3-methyladipic acid; 2, $p$-hydroxybenzoic acid; 3, $p$-hydroxyphenylacetic acid; 4, suberic acid; 5, $\alpha$-isosaccharino-1,4-lactone; 6, $\beta$-isosaccharino-1,4-lactone; 7; $p$-(n-amyl)benzoic acid (internal standard), and 8, homovanillic acid

tone in the ultrafiltrate was two- to threefold that of $\alpha$-isosaccharino-1,4-lactone.

**Identification of Isosaccharino-1,4-lactone in Normal Human Urine**

To determine if isosaccharino-1,4-lactone is normally present in human urine, we examined the ethyl acetate extract from normal human urine by gas chromatography/mass spectrometry. Figure 5 shows the total ion monitoring chromatogram of the trimethylsilylated extract. Peaks 5 and 6 showed the high ion peaks at $m/z$ 348 with relative intensities 78 and 100%, respectively, on their mass spectra. The mass spectra, however, were not identical with that of the pure trimethylsilylated isosaccharino-1,4-lactone, suggesting that peaks 5 and 6 contain other compounds. The ethyl acetate extract from normal human urine was then subjected to thin-layer chromatography and the eluate from the band corresponding to $\alpha$-$D$-isosaccharino-1,4-lactone was examined by gas chromatography/mass spectrometry as described. Figure 6 shows the total ion monitoring chromatogram of the trimethylsilylated eluate. Electron impact ionization mass spectra of peaks 5 and 6 in Figure 6 are shown in Figure 7. Although the relative intensities of the ions with small $m/z$ values were high as compared with those in Figures 3 and 4 because of higher ionizing energy (70 eV), peaks 5 and 6 had mass spectra identical with those of $\alpha$-$D$-isosaccharino-1,4-lactone and $\beta$-$D$-isosaccharino-1,4-lactone, respectively. Mass chromatograms at $m/z$ 348, 264, and 249 are shown in Figure 8; peaks 1 and 2 had retention times identical with $\alpha$-$D$-isosaccharino-1,4-lactone and $\beta$-$D$-isosaccharino-1,4-lactone, respectively. These pieces of evidence verify the identification of $\alpha$- and $\beta$-isosaccharino-1,4-lactone in normal human urine. The amount of the latter normally appearing
Fig. 7. Electron impact ionization (EI) mass spectrum of peak 5 (upper spectrum) in Fig. 6 and of peak 6 (lower spectrum) in Fig. 6.

Spectra were recorded under the following conditions: ionizing energy 70 eV, ionization current 300 μA, interface temperature 240 °C, ion source temperature 210 °C, and accelerating voltage 3 kV.

Fig. 8. Mass chromatograms at m/z 348, 264, and 249, and total ion monitoring (TIM) chromatogram of the trimethylsilylated fraction of normal human urine.

The sample and the analytical conditions were the same as in Figs. 6 and 7. Peak identifications: 1, α-isosaccharino-1,4-lactone; 2, β-isosaccharino-1,4-lactone; 3, p-(n-amy)lbenzoic acid (internal standard)

in human urine was about threefold that of the former (Figure 8). In human urine, isosaccharino-1,4-lactone was present at a much lower concentration than other organic acids such as 3-methyladipic acid, p-hydroxybenzoic acid, p-hydroxyphenylacetic acid, suberic acid, and homovanillic acid (Figure 5).
Table 3. Concentration of Isosaccharino-1,4-lactone in Normal Serum

<table>
<thead>
<tr>
<th>Peak-height ratio (m/z 348/m/z 249</th>
<th>Concentration of the lactone in serum, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.064</td>
<td>1.4</td>
</tr>
<tr>
<td>0.10</td>
<td>2.2</td>
</tr>
<tr>
<td>0.036</td>
<td>0.78</td>
</tr>
<tr>
<td>0.25</td>
<td>5.4</td>
</tr>
<tr>
<td>0.082</td>
<td>1.8</td>
</tr>
<tr>
<td>0.037</td>
<td>0.82</td>
</tr>
<tr>
<td>0.11</td>
<td>2.5</td>
</tr>
<tr>
<td>0.037</td>
<td>0.82</td>
</tr>
<tr>
<td>0.037</td>
<td>0.82</td>
</tr>
<tr>
<td>0.017</td>
<td>0.38</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>1.7 (1.5)</td>
</tr>
</tbody>
</table>

* 10 subjects; first seven are men.

**Interpretation of ¹H-NMR Spectra**

Figure 10d shows the entire ¹H-NMR spectrum of α-D-isosaccharino-1,4-lactone in pyridine-d₅. C₃-methylene protons appear as two quartets at the highest field, indicating that the two protons are not equivalent and are coupled with C₄-methylene proton. The region from 3.8 to 4.6 ppm contains the overlapped resonances of C₅-methylene and C₂'-methylene protons. The double resonance technique shows that C₅-methylene protons give rise to a multiplet through coupling with C₄-methylene proton and long-range coupling with C₃-methylene protons. C₂'-methylene protons give rise to two doublets, indicating that the methylene protons are not equivalent. A multiplet at 5.1 ppm is assigned to C₄-methylene proton and the multiplicity arises by unequal coupling of the...
methine proton with four other protons. Three hydroxy protons appear as a wide peak at 6.7 ppm.

We measured $^1$H-NMR spectra of α-D-isosaccharino-1,4-lactone in $^3$H$_2$O at different pD as in Figure 10 (a, b, c). At pD 1, C$_3$-methylene protons appear as a doublet through coupling with C$_4$-methylene proton, indicating that the methylene protons are apparently equivalent. At pD 7.4, C$_3$-methylene protons appear as two doublets, suggesting a small difference in chemical shifts of the methylene protons. The resonances of C$_3$-methylene protons show an upfield shift and give rise to two quartets at pD 11. C$_2'$-methylene protons give rise to a singlet at pD 1 and pD 7.4, while at pD 11 C$_2'$-methylene protons appear as two doublets. Clearly, some conformational change of α-D-isosaccharino-1,4-lactone is occurring in aqueous alkaline solution.

**Discussion**

Although we could determine the concentration of isosaccharino-1,4-lactone in serum by a mass fragmentographic technique, its concentration in urine could not be determined because some other compound seemed to interfere with the assay. In normal serum it is a major component of the acidic fraction, but its concentration in urine is low as compared with other organic acids. Apparently these organic acids are actively excreted into the urine, possibly by a renal tubular secretion mechanism, while isosaccharino-1,4-lactone seems to be excreted into the urine only by glomerular filtration.

The pH of a solution of α-D-isosaccharino-1,4-lactone in distilled water at a concentration of 1 g/L is 4.52, indicating that approximately 0.44% of α-D-isosaccharino-1,4-lactone has converted into α-D-isosaccharinate (Figure 11). One-milliliter aliquots of ultrafiltrate of serum, analyzed without pH adjustment, showed much lower concentrations (<10%) as compared with those determined with pH adjustment to 1. These findings suggest that in physiological fluid isosacccharino-1,4-lactone is present in its hydrated form, namely, isosaccharinate.

Isosaccharino-1,4-lactone originates at least partly endogenously, because it was detected in human serum after overnight fasting. Its physiological significance is not known. Work is in progress to elucidate its metabolism in the human organism.

We are grateful to Drs. Naohiro Shirai and Toyo Kaya (Nagoya City University) for their assistance in obtaining NMR spectra and for their valuable advice. We also thank Misses Masae Ishiguro and Tomiko Tanaka for their excellent technical assistance.

**References**