[Chem. Pharm. Bull. 18(12)2535—2543(1970)]

UDC 547.455.03.08 : 543.544.25

# Simultaneous Determination of Urinary Uronic Acids and Saccharic Acids by Gas Chromatography

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(Received July 13, 1970)

A simultaneous determination by gas chromatography of urinary uronic acids and saccharic acids is described.

Uronic acids are converted into corresponding stable aldonic acids with sodium borohydride, and contaminative basic, aromatic and neutral substances are removed with columns of Amberlite CG-120, XAD-2 and CG-400. After trimethylsilylation of carboxyl and hydroxyl groups, these acids are separately determined by gas chromatography.

By this method, D-glucuronic acid and D-glucaric acid were determined and moreover, free D-gluconic acid and alkali-labile glucuronides were analyzed from normal human urine.

An oxidative metabolic pathway of D-glucuronolactone to D-glucaric acid in mammals was demonstrated by Marsh,[2] Aarts[3] and Matsui, *et al.*[4] D-Glucaro-1,4-lactone, one of D-glucaric acid analogue, which is excreted in urine is known to be the competitive inhibitor of $\beta$-glucuronidase.[5] We were interested in the metabolism of D-glucuronolactone in mammalian systems and examined a useful method for the simultaneous determination of urinary D-glucuronic acid and D-glucaric acid by gas chromatography.

Previously the quantitative analysis of free D-glucuronic acid in urine has been studied by many investigators.[6–10] An enzymic method[2a] and chemical methods[11] for the quantitative analysis of D-glucaric acid in urine were reported.

For gas chromatography each uronic acid (D-glucuronic acid) gave several peaks resulting from anomeric and ring isomers and often accurate determination was not achieved.[12,13] To prevent multiplicity of peaks, uronic acids were converted into corresponding stable aldonic acids (L-gulonic acid) by the reduction using sodium borohydride. Gas chromatographic separation of aldonolactones derived by the lactonization of aldonic acids as their trimethyl-

1) Location: *Hongo-7-chome, Bunkyo-ku, Tokyo.*

2) *a*) C.A. Marsh, *Biochem. J.*, **86**, 77 (1963); *b*) *Idem, ibid.*, **87**, 82 (1963); *c*) *Idem, ibid.*, **89**, 108 (1963); *d*) *Idem, ibid.*, **99**, 22 (1966).
3) E.M. Aarts, *Biochem. Pharmacol.*, **14**, 359 (1965).
4) *a*) M. Matsui, M. Okada and M. Ishidate, *Chem. Pharm. Bull.* (Tokyo), **17**, 1064( 1969); *b*) M. Matsui, T. Kaizu, M. Okada and M. Ishidate, *ibid.*, **17**, 1871 (1969); *c*) M. Okada, M. Matsui, T. Kaizu and F. Abe, *ibid.*, **17**, 2625 (1969).
5) G.A. Levvy, *Biochem. J.*, **52**, 464 (1952).
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8) H. Takabayashi, *Japan. J. Gastroenterol.*, **55**, 92 (1958).
9) S. Ogiya and H. Kataoka, *Yakugaku Zasshi*, **79**, 949 (1959).
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silyl (TMS) derivatives was found to be satisfactory.[14,15] But by the lactonization with hydrochloric acid saccharic acids (D-glucaric acid) gave several peaks resulting from lactone isomers (1,4-, 6,3- and di-lactone).[12] We have overcome the difficulties to establish a simultaneous determination of D-glucuronic acid and D-glucaric acid by opening their lactone rings with triethylamine (TEA) followed by trimethylsilylation.

## Experimental

**Materials**——Pyridine (GR; Kanto Chemical Co., Ltd.) was dried with NaOH. Triethylamine (EP; Daiichi Pure Chemicals Co., Ltd.) was purified by refluxing with phthalic anhydride followed by distillation. Hexamethyldisilazane (EP; Tokyo Kasei Kogyo Co., Ltd.), trimethylchlorosilane (EP; Tokyo Kasei Kogyo Co., Ltd.), ammonium carbonate (GR; Kanto Chemical Co., Ltd.) and sodium borohydride (GR; E. Merck AG) were used directly. Amberlite CG-120 ($H^+$) resin (type I) was conditioned by washing the resin with 2N HCl followed by repeated washing with distilled water. Amberlite CG-400 ($HCO_3^-$) resin (type I) was conditioned by washing the resin with 1N $NaHCO_3$ followed by repeated washing with distilled water. Amberlite XAD-2 resin was conditioned by crushing the resin to 100—200 mesh followed by washing with methanol, 2N NaOH, 2N HCl and distilled water. Uronic, aldonic and saccharic acids were commercial samples or prepared in the laboratory and their purities were established from the results of elemental analysis and melting point.

**Gas Chromatography**——Gas chromatography was performed on a Shimadzu GC-1C or GC-4APF gas chromatograph equipped with hydrogen flame ionization detector. The glass tube (2.0 or 1.5 m×4 mm *i.d.*) was packed with 2% GE XF-1105, 2% DC QF-1, 2% GE SE-52, 2% OV-1 or 5% NGS (neopentyl glycol succinate) on a support of Gas-Chrom P (80—100 mesh).

**Sample Preparation**——As shown in Chart 1, galactaric acid as an internal standard was added to urine (1 ml) and the solution was passed through a column (6×100 mm) of Amberlite CG-120 ($H^+$) ion-exchange resin (*ca.* 3 ml) and then a column of Amberlite XAD-2 resin (*ca.* 3 ml). To the combined effluent and washings from the two columns were added 4 drops of triethylamine (TEA) and the solution was allowed to stand for 20 min at room temperature. Uronic acids and aldoses in the solution were reduced to their corresponding aldonic acids and alditols, respectively by treatment with 3% $NaBH_4$ (1 ml). After the solution had been allowed to stand for 1 hr at room temperature, the excess $NaBH_4$ was destroyed by the addition of Amberlite CG-120 ($H^+$) ion-exchange resin and the solution was then passed through a small column of the same resin (*ca.* 2 ml). To the combined effluent and washings from the column were added 4 drops of TEA and the solution was allowed to stand for 5 min at 60°. The solution was evaporated to dryness under reduced pressure and boric acid was removed as volatile methyl borate by evaporating twice with dry methanol (each 4 ml). The residue was dissolved in distilled water (3—5 ml) and then the solution was passed through a column (6×150 mm) of Amberlite CG-400 ($HCO_3^-$) ion-exchange resin (*ca.* 4.5 ml). The effluent and washings (*ca.* 20 ml) containing the neutral materials were discarded. The eluate from the column with 1M $(NH_4)_2CO_3$ (20 ml) was evaporated to dryness at 80° under reduced pressure (water pump). The residue was dissolved in distilled water (1 ml) and a drop of TEA was added to the solution. After the solution had been allowed to stand for 5 min at 60°, the solution was evaporated to dryness and the residue was dried in a desiccator under vacuum (oil pump). The sample was treated with 4 drops of pyridine, 3 drops of hexamethyldisilazane and 2 drops of trimethylchlorosilane for 20 min at 60° and 1 $\mu$l of the reaction mixture was injected directly to the gas chromatograph.

On the other hand, as shown in Chart 2, to the eluate from the above column of Amberlite XAD-2 resin with 10% TEA in 50% methanol (10 ml) was added galactaric acid as an internal standard and the solution was evaporated to dryness. The residue was dissolved in distilled water (1 ml) and 1% $NaBH_4$ (1 ml) was added to the solution. After the solution had been allowed to stand for 1 hr at room temperature, the reaction mixture was treated with Amberlite CG-120 ($H^+$) ion-exchange resin and the solution was then passed through a small column of the same resin (*ca.* 2 ml). The combined effluent and washings were evaporated to dryness under reduced pressure and then evaporated twice with dry methanol (each 4 ml). The residue was dissolved in distilled water (1 ml) and 3 drops of TEA was added to the solution. After the solution had been allowed to stand for 5 min at 60°, the solution was evaporated to dryness and the residue was dried in a dessicator under vacuum. The sample was trimethylsilylated as described above and 1 $\mu$l of the reaction mixture was injected directly to the gas chromatograph.

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15) *a*) M. Matsui, M. Okada, T. Imanari and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **16**, 1383 (1968); *b*) C. Chen, T. Imanari, H. Yamamoto and T. Kwan, *ibid.*, **16**, 755 (1968).

## Result and Discussion

### Gas Chromatography of Aldonic Acids and Saccharic Acids

As uronic acids were unstable[8,16–20] throughout previous treatments for gas chromatographic analysis, we converted them into corresponding stable aldonic acids by reduction with sodium borohydride.

For gas chromatography the procedure including conversion of carboxyl groups into methyl esters with diazomethane and subsequent conversion of hydroxyl groups into TMS ethers or trifluoroacetates did not give single derivatives, because the equilibrium[21] between free acids and lactones of aldonic and saccharic acids existed at the period that free acids were prepared. On the other hand salts of carboxylic acids, which were not esterified with diazomethane, were easily trimethylsilylated. TMS esters were stable in the presence of excess of reagent, otherwise the trace of atmospheric moisture brought about their decomposition. Trimethylsilylation was achieved by general method[22,23] using pyridine, hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS). A quantitative mixture of L-gulonic acid, D-glucaric acid and pyrene was therefore treated with these reagent mixtures at 60° and the appearance of TMS derivatives of L-gulonic acid and D-glucaric acid was followed kinetically by comparing the ratio of each peak height to the peak height of pyrene. As shown in Fig. 1, trimethylsilylation was completed within about 10 minutes at 60° and did not require so inconveniently long reaction times as used by previous investigators.[23]

Use of bis(trimethylsilyl)acetamide in place of HMDS gave the almost same results, but in this case, uric acid in urine was also trimethylsilylated to interfere with the determination of sugars.

The resolution of TMS derivatives of aldonic acids and saccharic acids was examined on several columns and relative retention times are given in Table I. The satisfactory separation was achieved on 2% QF-1 or 2% XF-1105 under isothermal condition (Fig. 2).

The calibration curves for L-gulonic acid and D-glucaric acid using galactaric acid as an internal standard are shown in Fig. 3.

### Conversion of Uronic Acids into Aldonic Acids

It has been known that aldehyde groups, ester groups and lactone rings in uronic acids and in their derivatives were reduced to alcohols with excess of sodium borohydride.[24] For selective reduction of the aldehyde groups, therefore, the lactone rings should be previously hydrolyzed. And for the purpose, as a volatile base, TEA was used, and with it completion of the hydrolysis within about 10 minutes was confirmed by the hydroxamic acid reaction. The resulted triethylammonium uronates were reduced to corresponding aldonates with aqueous sodium borohydride quantitatively within about 30 minutes, as shown in Fig. 4. The calibration curve for D-glucuronic acid coincided with that for L-gulonic acid indicating quantitative conversion of the former into the latter (Fig. 3).

### Analysis of Uronic Acids and Saccharic Acids in Human Urine

Combining two procedures described above, we erected a method for the analysis of uronic acids and saccharic acids; the procedures are detailed in the experimental section. When

16) I.R. Siddiqui and C.B. Purves, *Can. J. Chem.*, **41**, 382 (1963).
17) M. Ishidate, M. Kimura and M. Kawata, *Chem. Pharm. Bull.* (Tokyo), **11**, 1083 (1963).
18) F.G. Fischer and H. Schmidt, *Chem. Ber.*, **92**, 2184 (1959).
19) K.P. Link and C. Nieman, *J. Am. Chem. Soc.*, **52**, 2478 (1930).
20) E. Stutz and H. Deuel, *Helv. Chim. Acta*, **41**, 1722 (1958).
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22) C.C. Sweeley, R. Bentley, M. Makita and W.W. Wells, *J. Am. Chem. Soc.*, **85**, 2497 (1963).
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evaporations were achieved under acidic conditions (in the presence of hydrochloric acid, formic acid *etc.*), glucuronides in urine were hydrolyzed to interfere with the determination of free D-glucuronic acid and acidic sugars were partially destroyed by decarboxylation *etc.* We

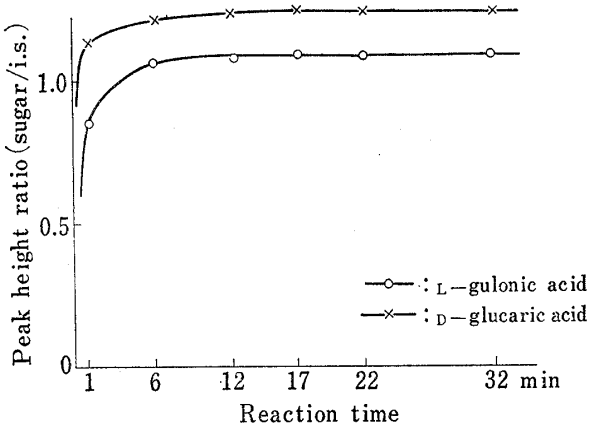


Fig. 1. Trimethylsilylation of L-Gulonic Acid and D-Glucaric Acid

L-Gulonolactone (100 μg) and D-glucaro-6,3-lactone (200 μg) were converted into triethylammonium L-gulonate and D-glucarate, and treated with 4 drops of pyridine, 3 drops of hexamethyldisilazane and 2 drops of trimethylchlorosilane at 60°. Pyrene was used as an internal standard (i.s.).
conditions: 2% XF-1105, 1.5 m×4 mm i.d., 170°, $N_2$ 65 ml/min

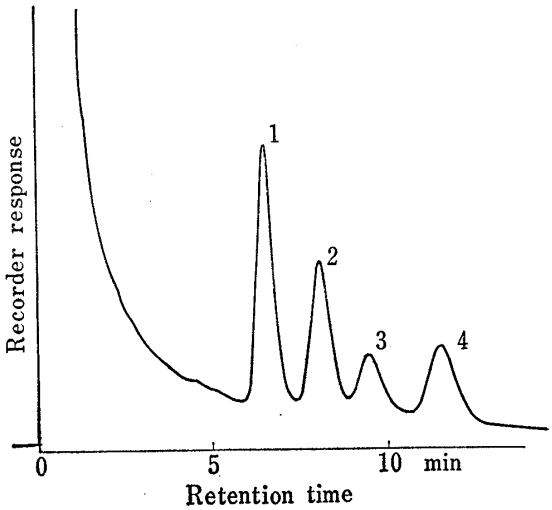


Fig. 2. Typical Gas Chromatogram of Aldonic Acids and Saccharic Acids as the Trimethylsilyl Derivatives

peak 1: L-gulonic acid 2: D-gluconic acid
3: D-glucaric acid 4: galactari cacid
conditions: 2% QF-1, 1.5 m×4 mm i.d., 120°, $N_2$ 60 ml/min

Table I. Relative Retention Times of Aldonic Acids and Saccharic Acids

| | A | B | C | D |
|---|---|---|---|---|
| Aldonic acid: | | | | |
| D-Erythronic acid | 0.112 | 0.098 | 0.118 | 0.130 |
| L-Threonic acid | 0.118 | 0.119 | 0.123 | 0.136 |
| D-Ribonic acid | 0.301 | 0.226 | 0.308 | 0.337 |
| L-Xylonic acid | 0.303 | 0.247 | 0.303 | 0.326 |
| D-Lyxonic acid | 0.319 | 0.249 | 0.321 | 0.352 |
| L-Arabinonic acid | 0.325 | 0.272 | 0.328 | 0.353 |
| D-Allonic acid | 0.697 | 0.506 | 0.731 | 0.777 |
| L-Gulonic acid | 0.698 | 0.484 | 0.723 | 0.762 |
| D-Mannonic acid | 0.707 | 0.490 | 0.729 | 0.777 |
| D-Altronic acid | 0.777 | 0.616 | 0.813 | 0.848 |
| D-Talonic acid | 0.780 | 0.611 | 0.805 | 0.844 |
| D-Galactonic acid | 0.793 | 0.624 | 0.819 | 0.849 |
| D-Gluconic acid | 0.813 | 0.625 | 0.840 | 0.870 |
| L-Idonic acid | 0.862 | 0.731 | 0.882 | 0.930 |
| Saccharic cid: | | | | |
| Erythraric acid | 0.138 | 0.171 | 0.139 | 0.147 |
| D,L-Threaric acid | 0.174 | 0.278 | 0.174 | 0.175 |
| D-Mannaric acid | 0.707 | 0.550 | 0.729 | 0.754 |
| D-Glucaric acid | 0.870 | 0.775 | 0.882 | 0.896 |
| Galactaric acid | 1.00 (10.16 min) | 1.00 (17.3 min) | 1.00 (9.31 min) | 1.00 (9.15 min) |

A: 2% XF-1105, 1.5 m×4 mm i.d., 180°, $N_2$ 65 ml/min
B: 5% NGS, 1.5 m×4 mm i.d., 130°, $N_2$ 60 ml/min
C: 2% SE-52, 1.5 m×4 mm i.d., 185°, $N_2$ 80 ml/min
D: 2% OV-1, 1.5 m×4 mm i.d., 190°, $N_2$ 65 ml/min

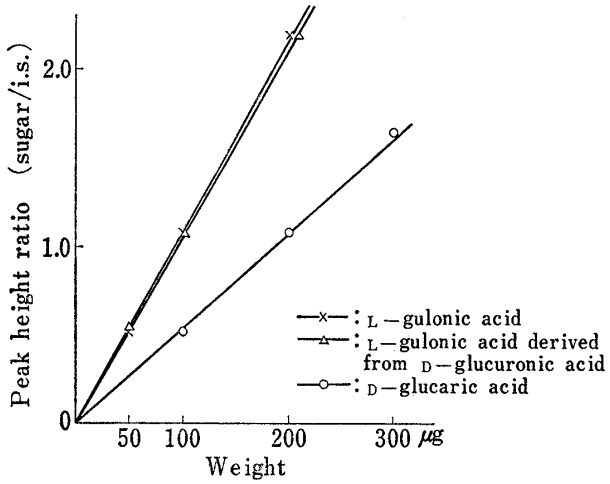


Fig. 3. Calibration Curves for L-Gulonic, D-Glucuronic Acid and D-Glucaric Acid using Galactaric Acid as an Internal Standard (i.s.)

300 μg of galactaric acid was used as an internal standard.
conditions: 2% XF-1105, 1.5 m×4 mm i.d., 170°, $N_2$ 65 ml/min

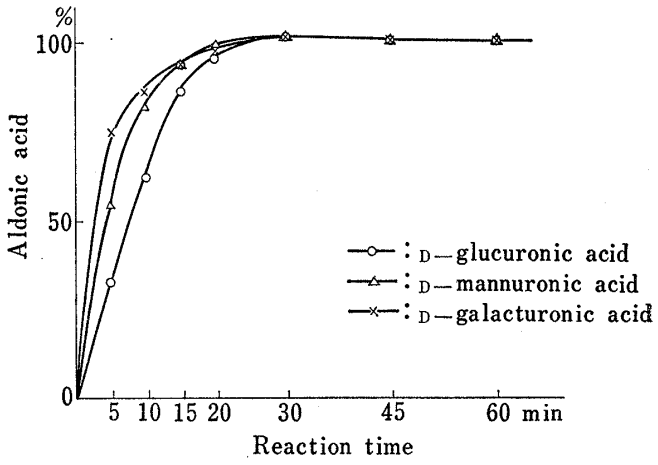


Fig. 4. Reduction of Uronic Acids with Sodium Borohydride

Each uronic acid (1 mg) was converted into the triethylammonium uronate and reduced with sodium borohydride (30 mg) in water (10 ml).
conditions: 2% XF-1105, 1.5 m×4 mm i.d., 170°, $N_2$ 65 ml/min
internal standard: D-glucitol

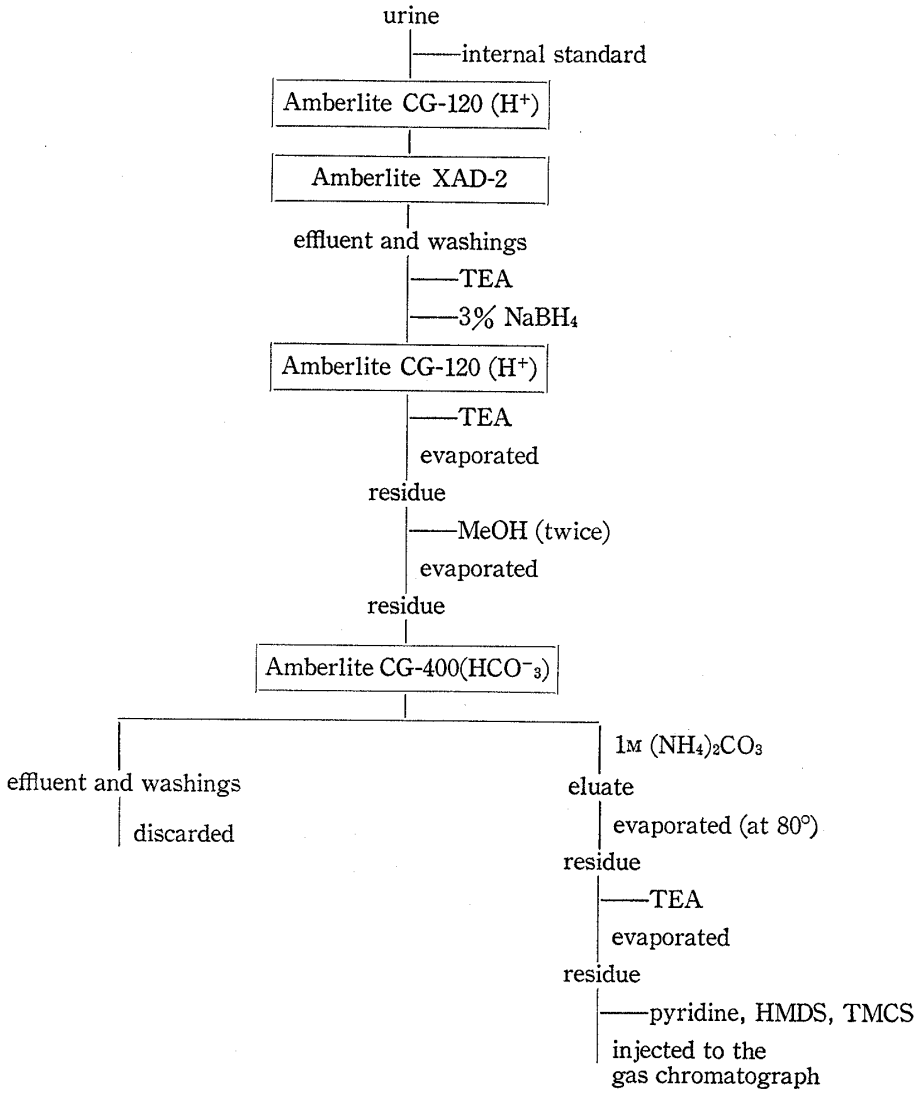


Chart 1. Analysis of Uronic Acids and Saccharic Acids in Human Urine

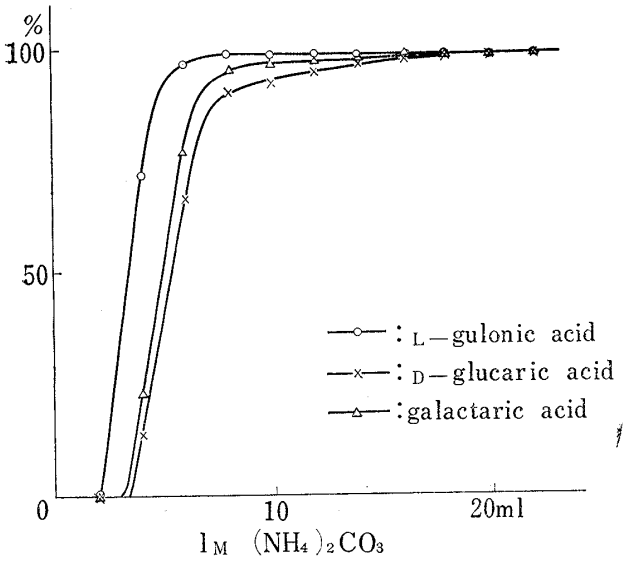


Fig. 5. Elution Curves of L-Gulonic Acid, D-Glucaric Acid and Galactaric Acid from the Amberlite CG-400 ($HCO_3^-$) Column with 1M Ammonium Carbonate

conditions: 2% XF-1105, 1.5 m×4 mm i.d., 170°, $N_2$ 65 ml/min
internal standard: D-glucitol

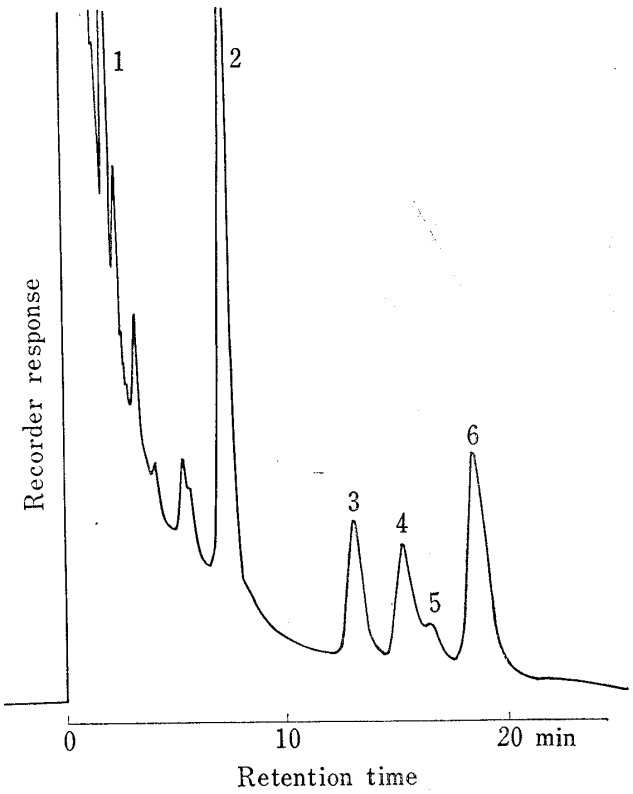


Fig. 6. Gas Chromtmarogram of Acidic Compounds in Human Urine

peak 1: erythronic acid 2: citric acid
3: L-gulonic acid[a] 4: D-gluconic acid
5: D-glucaric acid 6: i.s. (galactaric acid)
conditions: 2% XF-1105, 2.0 m×4 mm i.d., 170°, $N_2$ 65 ml/min
*a*) from D-glucuronic acid

Table II. Stability of L-Gulonic Acid, D-Glucaric Acid and Galactaric Acid in 1M Ammonium Carbonate during Evaporation at 80°

| No. | Recovery (%) | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | Average |
| L-Gulonic acid | 100.3 | 99.4 | 99.3 | 97.2 | 97.1 | 98.7 |
| D-Glucaric acid | 98.0 | 95.8 | 94.8 | 95.1 | 94.5 | 95.6 |
| Galactaric acid | 97.0 | 95.7 | 96.1 | 95.0 | 95.9 | 95.9 |

conditions: 2% XF-1105, 1.5 m×4 mm i.d., 180°, $N_2$ 65 ml/min
internal standard: D-glucitol

observed that saccharic acids were more unstable than uronic acids under acidic conditions. Therefore all evaporations were carried out under mild alkaline conditions, as shown in Chart 1. After passing urine sample through a cation-exchange resin column and then a polystyrene resin (Amberlite XAD-2) column, uronates were quantitatively converted into their corresponding aldonates. After removal of the sodium and triethylammonium ions from the reaction mixtures with cation-exchange resin, boric acid was removed by repeated evaporation with dry methanol. Aldonic and saccharic acids adsorbed on an anion-exchange resin ($HCO_3^-$ form) column were quantitatively eluted with 1M ammonium carbonate as shown in Fig. 5. Moreover they were stable during evaporation at 80° (Table II) and conveniently ammonium carbonate was decomposed to carbon dioxide and ammonia at this temperature.

A representative gas chromatogram of acidic sugars produced from urine by reduction with sodium borohydride are given in Fig. 6.

By omitting the reduction with sodium borohydride in Chart 1, the peak of L-gulonic disappeared, and consequently D-glucuronic acid was confirmed to be only one uronic acid present in normal human urine.

A satisfactory result was also obtained in the recovery test with human urine added with the difinite amounts of D-glucuronic acid and D-glucaric acid as shown in Table IV.

Table III. Relative Retention Times of Acidic Sugars in Human Urine

| | A | B | C | D |
|---|---|---|---|---|
| Standard: | | | | |
| L-Gulonic acid | 0.700 | 0.566 | 0.731 | 0.739 |
| L-Gulonolactone | 0.840 | — | — | — |
| D-Gluconic acid | 0.817 | 0.696 | 0.838 | 0.870 |
| D-Glucono-$\delta$-lactone | 0.592 | — | — | — |
| D-Glucono-$\gamma$-lactone | 0.636 | — | — | — |
| D-Glucaric acid | 0.875 | 0.821 | 0.890 | 0.873 |
| D-Glucuronic acid | 0.739, 0.978 | — | — | — |
| Galactaric acid | 1.00 (16.15 min) | 1.00 (11.47 min) | 1.00 (6.70 min) | 1.00 (11.12 min) |
| Acidic sugars produced from urine by reduction with $NaBH_4$ | 0.699<br>0.816<br>0.876 | 0.564[a)]<br>0.693<br>0.824 | 0.732<br>0.837<br>0.888 | 0.739<br>0.868 |
| Acidic sugars produced from urine by reduction with $NaBH_4$ followed by lactonization | 0.595<br>0.639<br>0.806 | — | — | — |
| Acidic sugars in urine | 0.740<br>0.815<br>0.875<br>0.979 | — | — | — |

A: 2% XF-1105, 2.0 m×4 mm i.d., 170°, $N_2$ 65 ml/min
B: 2% QF-1, 1.5 m×4 mm i.d., 120°, $N_2$ 60 ml/min
C: 2% SE-52, 1.5 m×4 mm i.d., 190°, $N_2$ 88 ml/min
D: 2% OV-1, 1.5 m×4 mm i.d., 170°, $N_2$ 65 ml/min
*a*) The peak of L-gulonic acid overlaps with that of citric acid in urine under the gas chromatographic condition described above.

Table IV. Recovery of D-Glucuronic Acid and D-Glucaric Acid Added to Human Urine

| No. | D-Glucuronic acid | | | | D-Glucaric acid | | | |
|---|---|---|---|---|---|---|---|---|
| | Added ($\mu$g) | Found ($\mu$g) Total | Diff. | Recovery (%) | Added ($\mu$g) | Found ($\mu$g) Total | Diff. | Recovery (%) |
| 1 | — | 28.6 | — | — | — | 9.8 | — | — |
| 2 | 50 | 75.3 | 46.7 | 93.4 | 300 | 300.8 | 291.0 | 97.2 |
| 3 | 100 | 123.9 | 95.3 | 95.3 | 200 | 220.0 | 210.2 | 105.1 |
| 4 | 200 | 224.0 | 195.4 | 97.7 | 100 | 113.4 | 103.6 | 103.6 |
| 5 | 300 | 338.7 | 310.1 | 103.4 | 50 | 62.0 | 52.2 | 104.4 |

conditions: 2% XF-1105, 2.0 m×4 mm i.d., 170°, $N_2$ 65 ml/min
internal standard: galactaric acid

Urinary levels of D-glucuronic acid and D-glucaric acid determined for 1 hr urines of normal men whose diets were restricted to rice only are shown in Fig. 7 and the values for D-glucaric acid are in good agreement with those determined by Ishidate, *et al.*[11a)] The values for D-

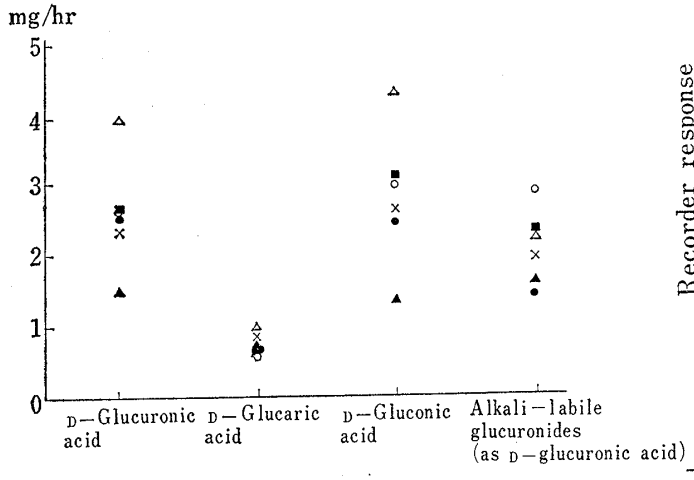


Fig. 7. Urinary Excretion of Free D-Glucuronic Acid, D-Glucaric Acid, D-Gluconic Acid and Alkali-labile Glucuronides in Normal Human Adults

conditions: 2% XF-1105, 2.0 m×4 mm i.d., 170°, $N_2$ 65 ml/min
internal standard: galactaric acid
●■▲: I (sex: M, age: 32) ○: M (M, 31)
×: K (M, 27) △: H (M, 24)

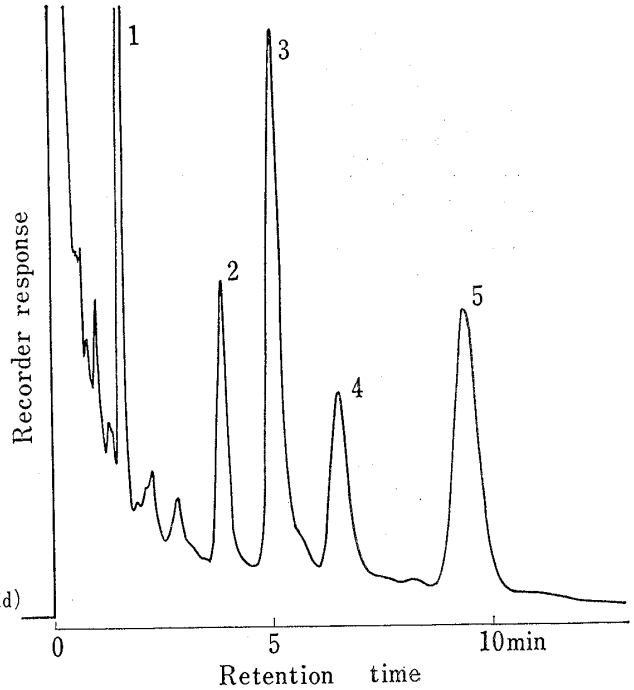


Fig. 8. Gas Chromatogram of the Fraction Eluted from the Amberlite XAD-2 Column

peak 1: *p*-hydroxybenzoic acid 2: unknown substance
3: hippuric acid 4: L-gulonic acid[a]
5: i.s. (galactaric acid)
conditions: 2% XF-1105, 1.5m×4 mm i.d., 170°, $N_2$ 65ml/min
*a*) from D-glucuronic acid

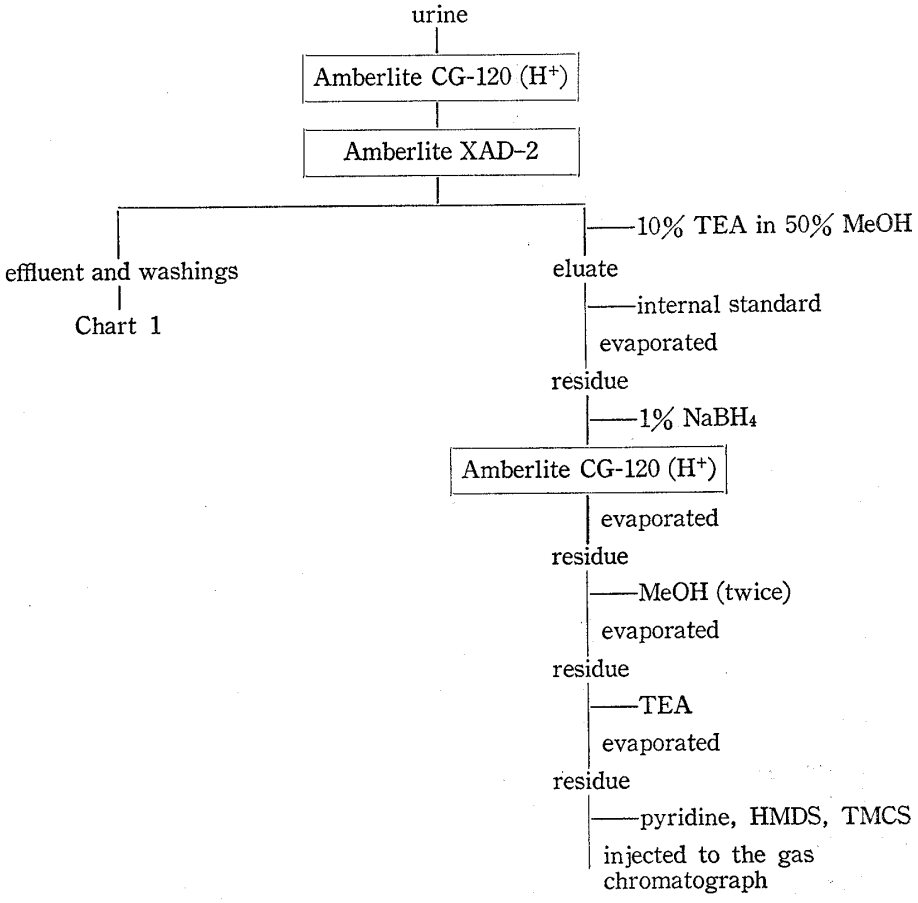


Chart 2. Analysis of Alkali-labile Glucuronides in Human Urine

glucuronic acid were not affected by addition of chemically stable glucuronides such as phenyl- and *p*-nitrophenyl-glucuronides, and as influential aliphatic ester-glucuronides and unstable N-glucuronides exist in negligible quantities in normal human urine, the values obtained by this method can be regarded as free D-glucuronic acid.

**Occurrence of D-Gluconic Acid in Normal Human Urine**

Excretion of free D-gluconic acid in normal human urine has been never reported. Peak 4 in Fig. 6 adjacent to the peak of D-glucaric acid (peak 5) was identified with the peak of D-gluconic acid on all columns, as summarized in Table III. Moreover, the peak was not changed by omitting the reduction with sodium borohydride. These results demonstrate that D-gluconic acid is excreted in normal human urine in a range of *ca.* 1—5 mg/hr as shown in Fig. 7.

**Alkali-labile Glucuronides Adsorbed on the Amberlite XAD-2 Column**

When the urinary adsorbate on the Amberlite XAD-2 column was eluted with 50% methanol, D-glucuronic acid was not detected in the eluate, which released the acid, however, by treatment with TEA. The facts indicated that some alkali-labile glucuronides were adsorbed on the column, and so, the glucuronic acid derived from such glucuronides was analyzed according to the procedure in Chart 2. A representative gas chromatogram of the fraction eluted from this column with 10% TEA in 50% methanol, are given in Fig. 8.

Since N-glucuronides occur in negligible quantities in normal human urine, the contents determined by this method are probably due to ester-glucuronides of bilirubin *etc.* (Fig. 7). A preliminary treatment of urine with alkali was found to approximately double the quantity of D-glucuronic acid determined by such method as described above. Therefore these alkali-labile glucuronides have been mis-measured as free D-glucuronic acid by the previous methods.[6–10]

**Acknowledgement** We thank Dr. M. Okada of Tokyo Biochemical Research Institute for his interest in our work.