[Chem. Pharm. Bull. 15(11)1677~1681(1967)]

UDC 615.739-07 : 547.544.25 : 547.483

# 213. Toshio Imanari and Zenzo Tamura : Gas Chromatography of Glucuronides.*1

(*Faculty of Pharmaceutical Sciences, University of Tokyo**2)

The applicability of gas chromatography to the analysis of various types of glucuronides including ether-, ester-, N- and S-glucuronides was studied by converting them to acetyl, methyl and trimethylsilyl derivatives.

(Received December 7, 1966)

Gas chromatography is a simple and rapid method of analysis which has been used successfully for various types of compounds. Separation and estimation of carbohydrates by this method are now practical laboratory procedures, but little attention has been paid to the gas chromatographic analysis of glucuronides. This study was undertaken to exmine the applicability of gas chromatography to the analysis of various types of glucuronides including ether-, ester-, N- and S-glucuronides, by converting them to the volatile derivatives. In order to prepare the derivatives, carboxyl group in glucuronic acid moiety was methylated with diazomethane prior to acetylation or trimethylsilylation, which was carried out in the usual manner. A microscale methylation procedure of glucuronides was achieved by the reaction with $CH_3I$ and NaH in dimethylformamide (DMF). The validity of the method was further verfied by applying it successfully to identification and determination of metabolites of *trans*-$\pi$-oxocamphor in urine.

## Experimental

**Apparatus**——Shimadzu Gas Chromatograph GC-1B (dual column type) equipped with hydrogen flame ionization detector was used. The carrier gas was nitrogen. A stainless steel tube (1.5 m. length) was packed with SE-30, QF-1, CNSi, SE-52 or NGS coated on Anakrom (90~100 mesh) or Gas Chrom P (60~80 mesh). Columns were made following to the filtration method by Horning.[1]

**Procedure : Acetyl Derivatives (Ac, Me)**——To a solution of glucuronides (0.5~1 mg.) dissolved in 1 ml. of MeOH, $CH_2N_2$ solution in ether was added until the solution became yellow and evaporated to dryness. The residue was acetylated with acetic anhydride and pyridine (1:1).

**Trimethylsilyl Derivatives (TMS, Me)**——The glucuronides were esterified with diazomethane as described above and trimethylsilylated with hexamethyldisilazane and trimethylchlorosilane in pyridine as reported by Sweeley, *et al.*[2]

**Methyl Derivatives (Me)**——To a solution of glucuronides (0.5~1 mg.) in 0.5 ml. of DMF, 0.2 ml. of NaH suspension in DMF was added under ice-cooling. After standing for 10 min. at room temperature with occasional shaking, 0.5 ml. of $CH_3I$ was added and the reaction mixture was kept further for 20 min. One ml. of ice water was added under ice-cooling to decompose the excess reagents and the mixture was extracted with 3 ml. of $CHCl_3$. The organic phase was separated and washed with $H_2O$ several times, dried on anhyd. $Na_2SO_4$ and evaporated to dryness in the stream of $N_2$ gas. The residue was dissolved in a small amount of acetone and submitted to gas chromatography.

## Results and Discussion

### Ether-glucuronides

**Acetyl Derivatives**——Ether-glucuronides (ether-G) were chemically stable and were readilly converted to their derivatives. A representative gas chromatogram of acetyl derivatives are given in Fig. 1. Eluted substances corresponding to the peaks were

*1 Preliminary communication of this work : This Bulletin, **12**, 1386 (1964).

*2 Bunkyo-ku, Hongo, Tokyo (今成登志男, 田村善蔵).

1) David Glick : "Methods of Biochemical Analysis" Vol. 11, p. 69 (1963). Interscience Publisher (New York, London).

2) C.C. Sweeley, Ronald Bentley, M. Makita, W.W. Wells : J. Am. Chem. Soc., **85**, 2497 (1963).

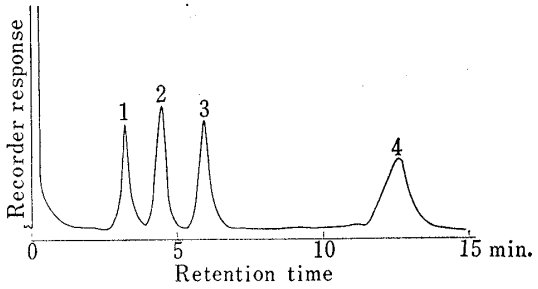


Fig. 1. Typical Gas Chromatogram of Ether-G (Ac, Me)

Peak: 1. phenol-G (Ac, Mc) 2. *p*-cresol-G (Ac, Me) 3. *p*-ethylphenol-G (Ac, Me) 4. *trans*-$\pi$-hydroxycamphor-G (Ac, Me)
column: 1.5% SE-30 on Gas-Chrom P, 1.5 m.×4 mm. *i.d.*
temperature: column 220°
sample heater 250°
detector 235°
carrier gas: $N_2$ 100 ml./min.
sens. 100 range 0.4 v.

collected and their IR spectra were compared with authentic materials. The result showed that the simple acetyl derivatives were fairly stable during gas chromatographic separation, although, as shown in Table I, *p*-nitrophenol-G (Ac, Me), $\beta$-naphthol-G (Ac, Me) and *trans*-$\pi$-hydroxycamphor-G (Ac, Me) are apt to be decomposed. They have longer retention times and are more unstable on a polar column than non-polar one. The reason of pyrolysis and the structure of pyrolytic products were studied and it was clarified that acetyl group of $C_4$ in glucuronic acid moiety was eliminated to make a double bond between $C_4$ and $C_5$.*3 Therefore, applicability of gas chromatography to acetyl derivatives may be limitted to the glucuronides of small and simple molecules.

Table I. Relative Retention Times of Ether-G (Ac, Me)

| Compound \ Stationary phase | 1.5% SE-30 | 2% QF-1 | 1% CNSi | 1% NGS |
|---|---|---|---|---|
| Phenol-G (Ac, Me) | 1.00*1 | 1.00*2 | 1.00*3 | 1.00*4 |
| *o*-Cresol-G (Ac, Me) | 1.22 | 1.10 | 1.23 | 1.08 |
| *m*-Cresol-G (Ac, Me) | 1.24 | 1.20 | 1.33 | 1.13 |
| *p*-Cresol-G (Ac, Me) | 1.36 | 1.36 | 1.37 | 1.24 |
| *p*-Ethylphenol-G (Ac, Me) | 1.76 | 1.57 | 1.80 | 1.68 |
| *p*-Nitrophenol-G (Ac, Me) | 3.64 | 7.65 | dec. | dec. |
| $\beta$-Naphthol-G (Ac, Me) | 5.12 | 5.38 | dec. | dec. |
| Butanol-G (Ac, Me) | 0.41 | 0.38 | 0.37 | 0.19 |
| Cyclohexanol-G (Ac, Me) | 0.88 | 0.80 | 0.85 | — |
| Methanol-G (Ac, Me) | 1.66 | 1.28 | 1.58 | 0.66 |
| Borneol-G (Ac, Me) | 1.79 | 1.44 | 1.68 | 0.79 |
| *trans*-$\pi$-Hydroxycamphor-G (Ac, Me) | 3.76 | 5.22 | 4.09 | dec. |

The values given are relative retention times with respect to phenol-G (Ac, Me), and retention times for it are 3.35, 4.75, 6.60 and 16.80 min. in *1, *2, *3 and *4 respectively.
column: 1.5 m.×4 mm. *i.d.* column temperature: 220° carrier gas: $N_2$ 100 ml./min.

**Methyl Derivatives**——In a preliminary experiment, the methylation of glucuronides following the procedure of Purdie[3] and Haworth[4] was found to be incomplete, since there have been observed several by-products in the gas chromatogram and the yield of these products varied depending on the reaction conditions. Recently, Hakomori[5] prepared methyl derivatives of glycolipids and polysaccharides with NaH and $CH_3I$ in dimethylsulfoxide (DMSO). Application of Hakomori's procedure to ether-G improved the reaction, but a small amount of by-product was accompanied. For example, the chromatogram of benzylalcohol-G methylated with this procedure showed a small peak following the main. The compound of the latter was identified as the completely

*3 The details will be published in This Bulletin.
3) T. Purdie, J.C. Irvine: J. Chem. Soc., **83**, 1021 (1903).
4) W.N. Haworth: *Ibid.*, **107**, 8 (1915).
5) S. Hakomori: J. Biochem., **55**, 205 (1964).

methylated glucuronide by IR spectrum showing an absorption band at 1755 $cm^{-1}$ assigned to C=O bond. On the other hand, the substance of minor peak has an absorption band at 1740 $cm^{-1}$ as shown in Fig. 2. Its structure was presumed as methyl (benzyl 4-deoxy-2,3-di-O-methyl-4,5-dehydro-β-D-glucopyranoside)uronate from the shift of C=O band, appearance of a new absorption band near 1655 $cm^{-1}$ assigned to C=C double bond and an absorption band at 230 mμ. The structure was confirmed by comparing the spectrum and the gas chromatogram with those of synthesized one through the way shown in Chart 1, where the deacetylation and methylation of methyl (benzyl 4-deoxy-2,3-di-O-acetyl-4,5-dehydro-β-D-glucopyranoside)uronate*3 were performed in the usual manner.

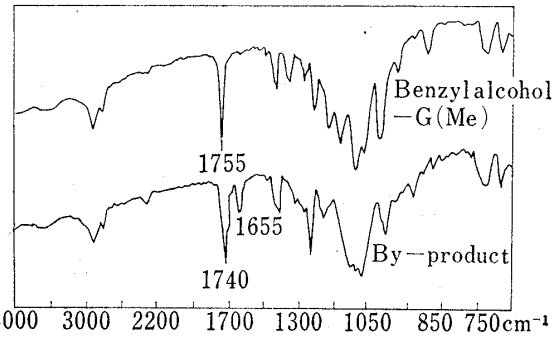


Fig. 2. IR Spectra of Benzyl Alcohol-G (Me) and By-product

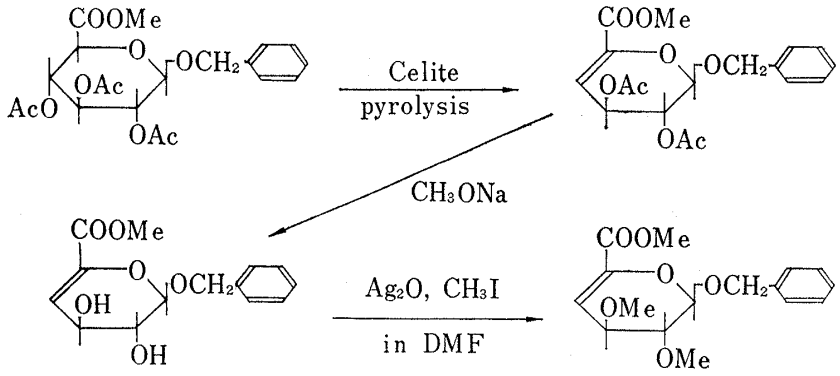


Chart 1.

A modification of Hakomori's method by using DMSO in place of DMF markedly suppressed the side-reaction, even where a large excess of NaH was undesirable. The methyl derivatives are more volatile and stable than the acetyl ones, as shown in Table I and II, and are favorable for further application to high molecular glucuronides.

Table II. Relative Retention Times of Methyl and Trimethylsilyl Derivatives

| Compound \ Stationary phase | | 1.5% SE-30 | 1.5% SE-52 | 2% QF-1 | 1% CNSi | 1% NGS |
|---|---|---|---|---|---|---|
| Phenol-G | Me | 1.00*1 | 1.00*2 | 1.00*3 | 1.00*4 | 1.00*5 |
| | TMS | 2.64 | 2.36 | 1.91 | 2.49 | 0.84 |
| *p*-Nitrophenol-G | Me | 4.03 | 3.86 | 7.44 | 5.06 | 8.84 |
| | TMS | 10.00 | 10.64 | 12.36 | 12.87 | 6.59 |
| Benzylalcohol-G | Me | 1.32 | 1.29 | 1.24 | 1.31 | 1.27 |
| | TMS | 3.34 | 3.20 | 1.93 | 3.20 | 1.03 |
| Cyclohexanol-G | Me | 0.82 | 0.81 | 0.78 | 0.81 | 0.51 |
| | TMS | 2.32 | 2.11 | 1.25 | 2.13 | 0.51 |
| Borneol-G | Me | 1.64 | 1.61 | 1.55 | 1.62 | 0.83 |
| | TMS | 4.08 | 3.71 | 2.18 | 3.71 | 0.78 |
| *trans*-π-Hydroxycamphor-G | Me | 3.64 | 3.61 | 6.09 | 4.15 | 4.06 |
| | TMS | 8.27 | 8.21 | 8.64 | 9.34 | 3.11 |

The retention times for phenol-G (Me) are 1.10, 1.40, 0.55, 1.67 and 1.57 min. *1, *2, *3, *4 and *5 respectively. conditions: the same as Table I.

**TMS Derivatives**——The derivatives are prepared simply and quantitatively. As shown in Table II, the TMS derivatives have longer retention times than the methyl derivatives on non-polar columns, but shorter on polar one such as NGS.

## S-glucuronides

Acetyl derivatives of thiophenol- and ethylmercaptan-G were chromatographed safely and their retention times are listed in Table III comparing with phenol-G (Ac, Me).

Table III. Retention Times

| Compound \ Stationary phase | 1.5% SE-30 (min.) | 1% CNSi (min.) | 1.5% SE-52 (min.) | 1% NGS (min.) |
|---|---|---|---|---|
| Ethylmercaptan-G (Ac, Me) | 1.30 | 2.22 | 1.33 | 3.75 |
| Thiophenol-G (Ac, Me) | 4.20 | 8.00 | 4.70 | 18.15 |
| Phenol-G (Ac, Me) | 2.91 | 5.62 | 3.19 | 12.81 |

column: 1.5 m. × 4 mm. *i.d.* column temperature: 220° carrier gas: $N_2$ 120 ml./min.

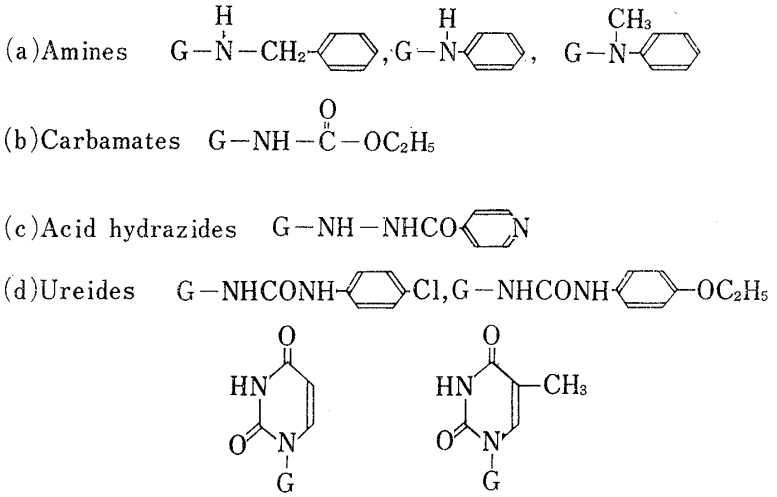


Chart 2.

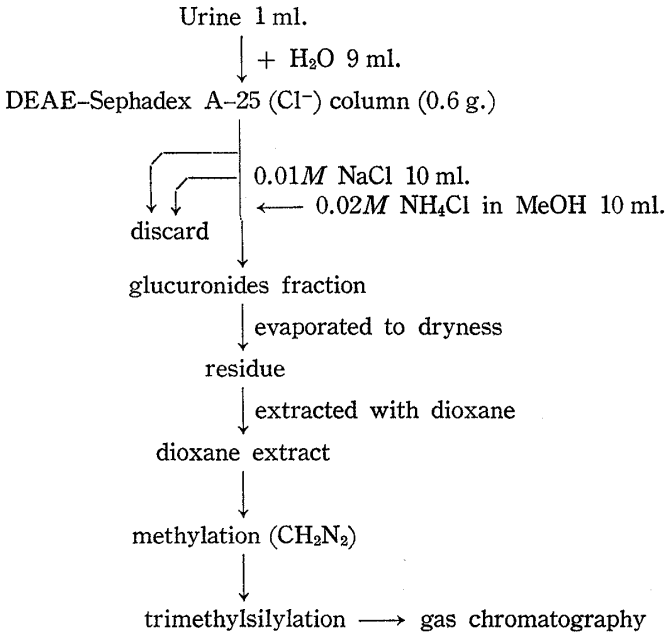


Chart 3. Separation of Glucuronides from Human Urine

Methyl and trimethylsilyl derivatives may also be prepared simply and chromatographed easily because S-glucuronides are chemically stable as ether-G.

## Ester-glucuronides

Among chemically synthesized acetyl derivatives of acetic acid-, isoketopinic acid- and benzoic acid-G, the former was fairly stable, while the latters were decomposed on columns and gave the characteristic gas chromatogram, in which some peaks were observed before main peak. Consequently ester-G (Ac, Me) will be unsuitable for gas chromatographic analysis. As ester-G without protecting groups has not been obtained synthetically,

the glucuronide of isoketopinic acid was obtained from urine of man administered with the acid. The methylation of the glucuronide was not successful, while the trimethylsilylated compound gave a single peak on the gas chromatogram.

**N-glucuronides**

N-glucuronides described in Chart 2 were examined. They are so unstable chemically and thermally that preparation of derivatives generally difficult. Exceptionally, glucuronides of uracil and thymine are stable and their methyl and TMS derivatives showed the single peak on gas chromatogram.

**Application of Gas Chromatography for Analysis of Glucuronides in Human Urine**

Essential prerequisite for analysis of glucuronides in biological materials by gas chromatography is separation from water and contaminants as well as quantitative formation of stable and volatile derivatives. Although various procedure had been presented for separation of glucuronides, none of them proved satisfactory for quantitative gas chromatographic analysis. We studied the purification of glucuronides from urine of a human administered *trans*-$\pi$-oxocamphor orally and the procedure in Chart 3 was found to be applicable. Twenty mg. of *trans*-$\pi$-oxocamphor was given to a man orally and his urine was collected at 5 hours administration. The sample was analysed by the procedure described above and the gas chromatogram of Fig. 3 was obtained.

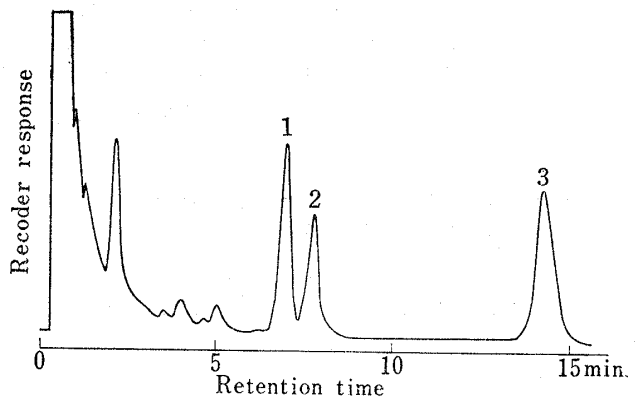


Fig. 3.

Peak : 1. *trans*-$\pi$-hydroxycamphor-G (TMS, Me) 2. isoketopinic acid-G (TMS, Me) 3. *p*-nitrophenol-G (TMS, Me)
column : 1% NGS on Anakrom, 1.5m.×4mm. *i.d.*
temperature : column 220°
sample heater 260°
detector 230°
carrier gas : $N_2$ 70 ml./min.
sens. 100 range 0.4 v.

This work was supported partially by a grant from the Iatrochemical Foundation to which the authors thanks are due. The authors are indebted to Prof. M. Akagi, Hokkaido University, and Chugai Pharmaceutical Co., Ltd. for supplying glucuronides.