[Chem. Pharm. Bull. 16(4) 613-617 (1968)]

UDC 543.854 : 543.544.25 : 581.19

Quantitative Analysis of Sugar Components in Steroidal Saponins by Gas Liquid Chromatography[1]

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(Received March 30, 1967)

Gas chromatographic determination of monosaccharides such as L-arabinose, L-rhamnose, D-xylose, D-mannose, D-galactose, and D-glucose was studied. The recovery of sugars under hydrolysis condition was examined by GLC (Fig. 1) and was found to be almost quantitative when steroidal glycosides were hydrolyzed with N sulfuric acid in dioxane-water (1:3) solution (Table III). A simple and accurate method by GLC was thus presented for the individual determination of sugar components in the micro amounts (5—10 mg) of steroidal saponins.

The molar ratios of sugar components were determined on the new convallasaponins by this method (Table V).

Although the paper chromatographic separation followed by colorimetric method[3] is often carried out for the analysis of sugar components in steroidal glycosides and polysaccharides, a more rapid as well as accurate method has been required for the studies in these fields. Since Sweeley, *et al.*[4] provided a valuable procedure for the gas chromatography (GLC) of sugars which should, in advance, be derived to the trimethylsilyl ethers, the applications for the quantitative analysis have also been developed.[5] The present authors found seven new steroidal saponins, convallasaponin-A,[8] -B,[7] -C,[8] -D,[7] -E,[6] glucoconvallasaponin-A,[6] and -B[7]; they contain L-arabinose, L-rhamnose, D-xylose and/or D-glucose. During the course of these studies, several quantitative methods using GLC were reported by other investigators for the sugar components in some natural substances.[9] However, no report was found on the gas chromatographic determination for the mixture of L-arabinose and L-rhamnose and on the damage of free sugars formed during the hydrolysis of their glycosides. Present paper deals with the method for the determination of sugar components in steroidal saponins and in other glycosides by GLC and with the application for some new convallasaponins.

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1) This paper represents Part IX of the series: "Constituents of Convallaria." Part VIII: *Chem. Pharm. Bull.* (Tokyo), **15**, 1713 (1967).
2) Location: *Nishi-6-chome, Kita-12-jo, Sapporo.*
3) E. Borel, F. Hostettler, and H. Deuel, *Helv. Chim. Acta*, **35**, 115 (1952); J.E. Jeffery, E.V. Partlow, and W.J. Polglase, *Anal. Chem.*, **32**, 1774 (1960); T. Momose, Y. Mukai, and M. Watanabe, *Talanta*, **5**, 275 (1960).
4) C.C. Sweeley, R. Bentley, M. Makita, and W.W. Wells, *J. Am. Chem. Soc.*, **85**, 2497 (1963).
5) M. Makita and W.W. Wells, *Anal. Biochem.*, **5**, 523 (1963); C.C. Sweeley and B. Walker, *Anal. Chem.*, **36**, 1461 (1964); J.M. Richey, M.G. Richey, and R. Shraer, *Anal. Biochem.*, **9**, 272 (1964); W.W. Wells, T. Chin, and B. Weber, *Clin. Chim. Acta*, **10**, 352 (1964); J.S. Sawardeker and J.H. Sloneker, *Anal. Chem.*, **37**, 945 (1965).
6) M. Kimura, I. Yoshizawa, and M. Tohma, *Chem. Pharm. Bull.* (Tokyo), in press.
7) I. Yoshizawa, M. Tohma, and M. Kimura, *Chem. Pharm. Bull.* (Tokyo), **15**, 129 (1967).
8) M. Kimura, M. Tohma, and I. Yoshizawa, *Chem. Pharm. Bull.* (Tokyo), **14**, 50 (1966); *ibid.*, **14**, 55 (1966).
9) G. Wulff, *J. Chromatog.*, **18**, 285 (1965); H.E. Brower, J.E. Jeffery, and M.W. Folsom, *Anal. Chem.*, **38**, 363 (1966).

Experimental

Materials

Stock solution: Aqueous solution was made in the concentration of 2 mg/ml for each of the following carbohydrates. L–rhamnose, L–arabinose, D–xylose, D–mannose, D–galactose, and D–glucose.

Internal standard solution: Aqeuous solution of mannitol in the concentration 2 mg/ml.

Reference glycoside: convallasaponin–C,[8] prosapogenin–C–I,[8] C–II[8] (from *Convallaria keisukei* Miq.), dioscin (from *Dioscorea* by Kawasaki, *et al.*[10]), and eupteleoside–A (from *Euptelea polyandra* Sieb et Zucc. by Goto, *et al.*[11]).

New convallasaponin: convallasaponin–A, –B, –D, prosapogenin–D–I, –II, –III, –IV, glucoconvallasaponin–A, and –B (from *Convallaria*[6]).

Gas Liquid Chromatography

Apparatus: Shimadzu model GC–1B with hydrogen flame ionization detector and dual columns was used.

Column: (a) 1.5% SE–30 on 60—80 mesh Chromosorb–W. 1.5 m × 4 mm *i.d.* U–shape stainless steel column. (b) 5% CNSi on 100—115 mesh Gas chrom–P. 2.25 m × 4 mm *i.d.* U–shape stainless steel column.

Column temperature: 140° for (a) and 120° for (b).

Flow rate: N_2 (30 ml/min) as carrier gas; H_2 (35 ml/min) and air (0.6 liter/min) for detector.

Relative Retention Times and Responses——A mixture of stock solution (1 ml) and internal standard solution (1 ml) was evaporated to dryness *in vacuo*. The residue was dissolved in dry pyridine (1 ml) and allowed to react with hexamethyldisilazane (0.2 ml) and trimethylsilyl chloride (0.1 ml) at room temperature for 5 min. Excess reagents and pyridine were evaporated from the reaction mixture *in vacuo* at 60°, which otherwise interfer the determination through tailing. After the residue was extracted with *n*–hexane (1 ml), the solution (1—5 μl) was injected for GLC. Relative retention times and responses of each sugar to mannitol as the internal standard were obtained as summarized in Table I and II, respectively. Calibration curve for each sugar was drawn up for such a series of volumes of the stock solution as 0.5, 1.5, 2.0, and 2.5 ml.

Stability of Sugars under Hydrolysis Conditions

Method 1: A mixture of the stock solution (5 ml) for each sugar, the internal standard solution (5 ml), 6 N HCl (5 ml), and EtOH (15 ml) was refluxed on a water–bath. After each elapse of times such as 0, 1, 2, 3, 4, and 5 hr, a portion (*ca.* 3 ml) of the reaction mixture was diluted with water, neutralized by Dowex–3, evaporated to dryness *in vacuo*. The residue was silylated and submitted to GLC for the determination of the sugar as described above; the results are shown in Fig. 1 and Table III.

Method 2: A mixture of the stock solution (5 ml) for each sugar, the internal standard solution (5 ml), 4 N H_2SO_4 (5 ml), and dioxane (5 ml) was heated in a water–bath at 95—100°. The rate of change was determined for each sugar in a similar way to that described above.

Determination of Sugar Components——Steroidal saponin (5—10 mg) was dissolved in a mixture of dioxane (1 ml), 2 N H_2SO_4 (2 ml), and the internal standard solution (1 ml), and was heated on a water–bath at 95—100° for about 5 hr[12] until the hydrolysis was completed, under the check by using thin–layer chromatography (solvent systems: 5 and 30% MeOH–$CHCl_3$). Water (4 ml) was then added to the reaction mixture, the aglycone separated was filtered off, the filtrate was neutralized by passing through the column of Dowex–3 (3 ml), the column was washed out with water (8 ml), and the combined aqueous solution was evaporated to dryness *in vacuo*. After the well-dried residue was silylated in a mixture of pyridine (1.0 ml), hexamethyldisilazane (0.2 ml) and trimethylsilyl chloride (0.1 ml) for 5 min at room temperature, the reaction mixture was evaporated to dryness under reduced pressure. The residue was extracted with *n*–hexane (0.5 ml) and the solution (1—5 μl) was submitted to GLC.

Calculation

The following equation gives the weight (S_W mg) of sugar:

$$S_W = \frac{S_S \cdot M_W}{M_S \cdot F_1 \cdot F_2}$$

where S_S: Total peak area of the anomeric sugars, obtained by the method of peak–width at half height.
M_S: Peak area of internal standard (mannitol).
F_1: Relative response of sugar.
F_2: Recovery of sugar.
M_W: Weight (mg) of internal standard added.

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[10] T. Kawasaki and T. Yamauchi, *Chem. Pharm. Bull.* (Tokyo), **10**, 703 (1962).

[11] M. Goto, S. Imai, T. Murata, and S. Fujioka, *Chem. Pharm. Bull.* (Tokyo), **12**, 516 (1964).

[12] 7 hours for dioscin.

Results and Discussion

Liquid phases such as SE-30, QF-1, CNSi, and NGS were examined for the gas chromatographic determination of six monosaccharides, L-arabinose, L-rhamnose, D-xylose, D-glucose, D-mannose, and D-galactose, which are common in the naturally occurring steroidal saponins. The column coated with these liquid phases such as QF-1 and NGS in concentration of two per cent were found to be unsuitable, owing to the tailing and the insufficient separation of peaks on the chromatogram. When SE-30 in concentration of 1.5 per cent was employed instead, the separation of L-rhamnose from both of L-arabinose and D-xylose was also unsatisfactory, contrary to the case of hexoses giving symmetrical peaks, as are summarized in Table I. The column coated with CNSi in concentration of five per cent, on the other hand, gave rather enough separation of pentoses to be determined quantitatively, in contrast to each pair of D-xylose and D-mannose and of D-glucose and D-galactose. The dual column using SE-30 and CNSi in the respective concentration of 1.5 and 5 per cent would, therefore, be suitable for the simultaneous determination of these monosaccharides described above. In anticipation of the simplicity and accuracy, the internal standard was employed and mannitol was found to be quite suitable, which on GLC gave only one peak standing far from these sugars and was sufficiently stable during the period of acid hydrolysis as stated below. Relative retention times and responses of the sugars to the internal standard are summarized in Table I and II, respectively. Calibration curves drawn by using these values showed good linearity having mean error of two per cent, within the range of weight-ratio of 0.50—2.50 to mannitol as a unit. Thus, such monosaccharides as described above could individually be estimated quantitatively by using GLC.

TABLE I. Relative Retention Times of Sugars

	1.5% SE-30 (140°)			5% CNSi (120°)		
L-Rhamnose	0.20	0.25		0.38	0.59	
L-Arabinose	0.19	0.21	0.24[a]	0.35[a]	0.48	0.65
D-Xylose	0.20[a]	0.30	0.38	0.70	0.90	
D-Mannose	0.51	0.84		0.72	1.42	
D-Galactose	0.55[a]	0.66	0.88	1.07	2.62	
D-Glucose	0.74	1.27		1.09	2.68	
Mannitol	1.00 (27.5 min)			1.00 (22.2 min)		

a) small peak
Sugars were trimethylsilylated beforehand.

TABLE II. Relative Responses of Sugars

Trimethylsilyl Sugar	1.5% SE-30	5% CN-Si
L-Rhamnose	1.04	0.98
L-Arabinose	1.04	1.01
D-Xylose	0.99	0.98
D-Mannose	0.99	1.01
D-Galactose	1.02	1.03
D-Glucose	0.99	1.00

Standard: mannitol

Glycosides have usually been hydrolyzed by refluxing with 1—2 N HCl in 50% ethanol, in order to obtain their aglycones. In the study of Dioscorea saponins, Tsukamoto, *et al.*[13]

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[13] T. Tsukamoto, T. Kawasaki, and T. Yamauchi, *Chem. Pharm. Bull.* (Tokyo), **4**, 35 (1956).

found that D-glucose and L-rhamnose were highly damaged under these conditions, recommending thus the following method of hydrolysis in two steps for the determination of the sugar components. According to them, after glycosides were heated in the dioxane-water (1:3) mixture containing N H_2SO_4 on a water-bath for three hours, the free sugar in the filtrate was determined as the first step and the insoluble substance remained was heated again for another three hours in the dioxane-water (1:3) mixture containing N HCl for the second determination. In the present study, the sugars were found to be so highly damaged under general condition of hydrolysis (Method 1) that forty per cent of the initial amount was lost as are shown in Fig. 1 and Table III. The strange peak on GLC appeared also in this case, interfering the analysis. On the other hand, when each sugar in the dioxane-water (1:3) mixture containing N H_2SO_4 was heated in a boiling water-bath to observe its stability during the elapse of times (Method 2), about ninety per cent or more of the initial amount was found to be recovered in good reproducibility as is summarized in Table III; the internal standard substance, mannitol, was completely stable under the condition. The hydrolysis of glycosides under the method 2 may, therefore, be highly suitable for the determination of their sugar components.

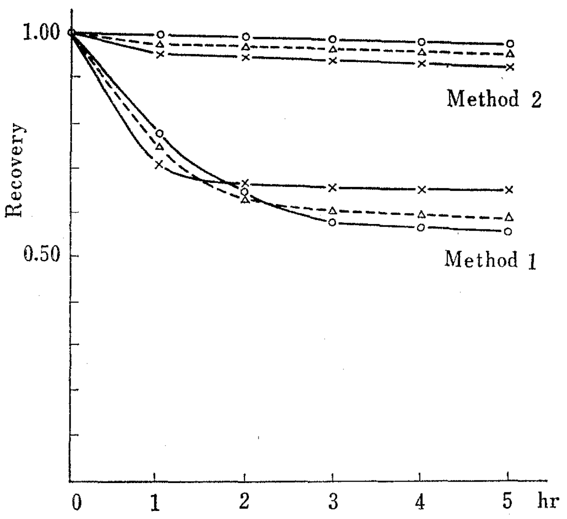

Fig. 1. Stability of Sugars under Hydroylsis Conditions

×—×—× L-arabinose
△···△···△ L-rhamnose
○—○—○ D-glucose

Table III. Recovery of Sugars under Hydrolysis Conditions

Sugar	Method 1	Method 2
L-Rhamnose	0.59±0.05	0.95±0.01
L-Arabinose	0.66±0.04	0.93±0.03
D-Xylose	—	0.94±0.02
D-Mannose	—	0.92±0.03
D-Galactose	—	0.89±0.03
D-Glucose	0.56±0.02	0.98±0.02
Mannitol	1.00	1.00

According to the gas chromatographic method described in the experimental part, the results obtained from the glycosides having known chemical constitutions such as convallasaponin-C, prosapogenin-C-I, -C-II, dioscin,[10] and eupteleoside,[11] were in good agreement with the theoretical value for each glycoside as summarized in Table IV, respectively. The same procedure has also been applied in the study of a series of new glycosides such as convallasaponin-A, -B, -D, prosapogenin-D-I, -D-II, -D-III, and -D-IV and the molar ratio of each sugar component was determined as summarized in Table V. Studies on the chemical constitutions of these glycosides have highly been accelerated by adopting this procedure; that will be reported in the forthcoming papers. It may be concluded from these results that the quantitative method presented here would be simple and accurate and be recommendable for the individual determination of sugar components in micro amounts of steroidal glycosides, triterpen saponins, and polysaccharides.

TABLE Ⅳ. Sugar Analysis of Reference Glycosides

Glycoside	Weight (mg)	Sugar	Mole	Calcd. (%)	Found (error)	
					1.5% SE-30	5% CN-Si
Convallasaponin-C[8]	6.31	ara	1	17.5	—	17.2(−1.7)
		rha	2	38.3	—	39.3(+2.6)
Prosapogenin-C-I[8]	6.44	ara	1	21.1	—	20.7(−2.8)
		rha	1	23.1	—	23.6(+1.3)
Prosapogenin-C-Ⅱ[8]	5.33	ara	1	26.6	25.8(−3.0)	26.1(−1.9)
Dioscin[10]	8.8	rha	2	37.8	37.1(−1.5)	37.5(−1.0)
		glc	1	20.7	20.5(−1.0)	20.8(+0.5)
Eupteleoside-A[11]	14.0	ara	1	16.1	16.0(−0.6)	16.0(−0.6)
		rha	1	17.6	16.9(−4.0)	17.2(−2.3)
		glc	1	19.4	19.3(−0.5)	19.5(+0.5)

TABLE V. Sugar Analysis of New Convallasaponins

Glycoside	Weight (mg)	Sugar	Mole	Calcd. (%)	Found (error)	
					1.5% SE-30	5% CN-Si
Convallasaponin-A	4.84	ara	1	25.7	24.5(−4.6)	25.6(+0.4)
Convallasaponin-B	5.31	ara	1	25.2	24.0(−4.7)	24.8(−1.5)
Convallasaponin-D	8.40	rha	2	32.3	31.7(−1.8)	33.1(+2.7)
		xyl	1	14.7	15.2(+3.4)	15.2(+3.4)
		glc	1	17.7	17.7(0)	17.6(−0.6)
Prosapogenin-D-I	7.01	rha	2	38.3	36.9(−3.6)	39.6(+3.3)
		xyl	1	17.5	17.5(0)	17.4(−0.6)
Prosapogenin-D-Ⅱ	5.44	rha	1	23.1	22.1(−4.3)	23.7(+2.6)
		xyl	1	21.1	21.0(−0.5)	21.2(+0.5)
Prosapogenin-D-Ⅲ	6.11	rha	1	28.4	27.5(−3.1)	29.1(+2.4)
Prosapogenin-D-Ⅳ	6.66	glc	1	30.3	30.6(+1.0)	30.2(−0.3)
Glucoconvallasaponin-A	4.81	ara	1	21.6	20.8(−3.7)	21.2(−1.8)
		glc	1	25.6	25.7(+0.4)	25.8(+0.8)
Glucoconvallasaponin-B	4.31	ara	1	19.8	19.2(−3.0)	20.0(+1.0)
		glc	1	23.6	23.6(0)	23.2(−1.6)

Acknowledgement The authors are indebted to Prof. T. Kawasaki, Kyushu University, and to Dr. M. Goto, Takeda Chemicals Co., Ltd., for the generous gift of dioscin and eupteleoside, respectively.