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EVALUATION OF A GAS CHROMATOGRAPHIC METHOD FOR THE QUANTITATIVE ESTIMATION OF HEXOSES FROM NEUTRAL GLYCOLIPIDS*,**

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SUMMARY

The alditol acetate method for the estimation of the hexoses from the carbohydrate moiety in neutral glycolipids has been standardized using gas–liquid chromatographic conditions which provided better resolution of the individual neutral sugars. The method involves the aqueous acid hydrolysis of the glycolipid, reduction of the released free sugars to the alcohols and acetylation before gas chromatographic analysis. Glucose-containing lipids differ from galactolipids in that the former require a longer time of hydrolysis for a quantitative cleavage of the sugar from the ceramide moiety. Results of the application of this method to glycolipids in the range of 0.2 to 0.5 μmole gave quantitative values using arabinose as internal standard. The sugar estimations of cerebrosides and sulphatides in the presence of phosphatides also gave quantitative results. The method has also been applied to determine the galactose to glucose ratio of ceramide di-, tri- and tetrahexosides of human erythrocyte ghost lipids.

INTRODUCTION

A number of gas chromatographic methods are available by which the sugars present in biological materials can be estimated[1]. The most important and commonly used of these are those of the trimethylsilyl[1,2], trifluoroacetyl[3] and alditol acetate[4] derivatives. Quantitative aspects of the former two methods have been fairly well explored, but systematic and quantitative studies on the estimation of the sugars from individual glycolipids as their alditol acetates are lacking. Such a study would be worth undertaking, as the alditol acetates provide a single peak in gas–liquid chromatography (GLC) thereby making quantitation easier. In the present paper, the quantitative estimation of galactose and glucose in neutral glycolipids by GLC of their alditol acetates is reported and the application to ceramide hexosides and other glycolipids is shown.

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** Arab = arabinose; Cer = ceramide; Cereb = cerebroside; Gal = galactose; Gal·NHAc = N-acetylgalactosamine; Glu = glucose.

EXPERIMENTAL

Materials

Standard carbohydrates (arabinose, galactose and glucose) were purchased from Merck, Darmstadt, G.F.R. and Fluka, Buchs, Switzerland (glucosamine hydrochloride and galactosamine hydrochloride). Pyridine, toluene, acetic anhydride, methanol, glacial acetic acid and hydrochloric acid were all analytical-grade reagents. Acetic anhydride and toluene were freshly distilled before use. The pyridine was refluxed for 1 h over potassium hydroxide pellets, distilled and stored besides potassium hydroxide pellets in a desiccator over anhydrous sulphuric acid. Sodium borohydride was obtained from Merck.

Gas-liquid chromatography

The liquid and stationary phases were purchased from Hewlett-Packard (Avondale, Pa., U.S.A.). All analyses were carried out with a Varian Aerograph Model 1800 gas chromatograph equipped with flame ionization detectors and a Type 481 digital integrator (Varian, Palo Alto, Calif., U.S.A.). Glass columns, 200 cm × 2 mm I.D., were packed with 1% OV-225 on Gas-Chrom Q. The operating conditions of the gas chromatograph are given in Table I.

Preparation of the alditol acetates

The alditol acetates of the individual carbohydrates were prepared and recrystallized by the method of Abdel-Akher *et al.*[5] and were used as test substances. In order to determine the response factor K, the carbohydrate samples were dried thoroughly in a drying pistol at 76° for 48 h over phosphorus pentoxide. All values were determined relative to arabinose, which also served as an internal standard for the estimation of neutral sugars from glycolipids. About 50–100 μg of each sugar with a similar amount of arabinose were taken up in 100 μl of distilled water and reduced with 100 μl of 0.8 M sodium borohydride solution in water for 2 h at room temperature. (Stock solutions of sodium borohydride in water were prepared every two weeks.) The reaction was stopped by the addition of two drops of acetic acid and the solution was evaporated with repeated additions of methanol–acetic acid (200: 1). Thorough drying was achieved by allowing the samples to stay overnight in a desiccator *in vacuo* over phosphorus pentoxide.

Acetylation was carried out with 300 μl of acetic anhydride at 100° for 2 h according to Metz *et al.*[6]. The acetic anhydride was removed by evaporation under vacuum with three 2-ml volumes of toluene. The alditol acetates were taken up in 2 ml of chloroform and washed with an equal volume of distilled water. After centrifugation the chloroform phase was separated, evaporated and dried. The residue was dissolved in 100 μl of acetone before GLC analysis.

Isolation of glycolipids

Large amounts of pure sphingolipids were prepared from crude polar lipid mixtures from normal human brain obtained by extraction according to Folch *et al.*[7] and subjecting the Folch lower-phase lipids to Dawson's[8] mild alkaline and acid hydrolysis. The crude glycolipids were then separated on a silicic acid column with stepwise elution with increasing volumes of methanol in chloroform[9]. The elution of various glycolipids was followed by thin-layer chromatography (TLC)[10].

Cerebroside fractions obtained directly from the total lipids (*e.g.*, rat and ox brain) by using Florisil column chromatography[11] were also used without further purification. They were thin-layer chromatographically pure, as examined on silica gel G plates with chloroform–methanol–water (65:25:4) as solvent system.

An enriched fraction of glycolipids obtained from human erythrocyte ghost lipids by adopting the method of Vance and Sweeley[12] was separated into mono-, di-, tri- and tetrahexosyl ceramides by preparative TLC according to Suzuki[13]. The ceramide dihexoside was also obtained from a pathological brain[14] by employing the above methods.

Monogalactosyl and digalactosyl diglycerides were gifts from Dr. Heinz of the Botanisches Institut and glucocerebroside from Dr. Tschöpe[15] of the Institut für physiologische Chemie of this university.

Mean molecular weight calculations were made for all the tested lipids by the analysis of fatty acids and sphingosine bases.

Hydrolysis of glycolipids

The glycolipid sample containing 50–100 μg of sugar was hydrolyzed with 1 ml of aqueous 1 *N* hydrochloric acid. Quantitative hydrolysis of galactolipids was achieved in 6 h at 100°. However, for the cleavage of the glucose–ceramide bond a 12-h hydrolysis was necessary. Therefore, as galactolipids did not show any loss in galactose content after 12 h of hydrolysis, in later experiments the 12-h hydrolysis was used (see Table V). After cooling about 50 μg of arabinose were added as an internal standard and the fatty acids were removed with three 2-ml volumes of petroleum ether (b.p. up to 57°) and two 2-ml volumes of chloroform. The aqueous phase was evaporated to dryness at 35° under nitrogen and the residue was dried overnight over potassium hydroxide pellets in a vacuum desiccator (to remove the hydrochloric acid). Further steps of reduction and acetylation were the same as for the free carbohydrate above. The alditol acetates were taken up in 100 μl of acetone and an aliquot was used for GLC.

RESULTS

Chromatographic resolution of alditol acetates of standard sugars

The neutral monosaccharides, such as glucose and galactose, can be isothermally analysed at 190°. For the elution of the amino sugars, it is necessary to programme the temperature to 226°, as given in Table I. The 1% OV-225 glass columns used in this study have recently been shown to give better resolution of the sugars than the ECNSS-M column used so far[6].

Determination of the response factor K

The response factor *K* was determined by either taking standard solutions of presynthesized alditol acetates or preparing standard solutions of free sugars and subjecting them to derivatization as given in Experimental. The *K* values were calculated exactly as reported by Sawardeker *et al.*[16] and the values obtained in a series of individual experiments are given in Table II.

Application to glycolipids

Application of the method (6-h hydrolysis) to the estimation of galactose in

TABLE I

GAS–LIQUID CHROMATOGRAPHIC CONDITIONS

Condition	*Value*
Column temperature	
Isothermal*	190°
Temperature programming**	Isothermal 190° (27 min) 2°/min (18 min) Isothermal (18 min)
Detector cell temperature	250°
Injector temperature	225°
Gas flow-rates, ml/min	
Hydrogen	20
Air	200
Nitrogen carrier gas	20
Sample volume, μl	0.1–0.5

* For neutral sugars.
** For neutral and amino sugars.

TABLE II

RESPONSE FACTOR K FOR PRE-SYNTHESIZED ALDITOL ACETATES AND FREE SUGARS AFTER CONVERSION TO ALDITOL ACETATES

$$K = \frac{\text{area of sugar peak} \times \mu\text{g arabinose}}{\text{area of arabinose peak} \times \mu\text{g sugar}}.$$

	Galactose	*Galactitol hexaacetate*	*Glucose*	*Sorbitol hexaacetate*
	1.060	0.972	0.985	0.958
	0.996	0.973	0.960	1.064
	0.983	0.973	0.999	0.998
	1.030	0.960	0.980	1.017
	0.987	0.996	1.026	1.024
		0.950	0.950	0.991
		0.969		0.977
Mean ± S.D.	1.011 ± 0.014	0.970 ± 0.005	0.983 ± 0.011	1.010 ± 0.010

samples of brain cerebrosides of different species is shown in Table III. The percentage recoveries for several galactose-containing lipids are summarized in Table IV. As mentioned above, the theoretical percentages required for the calculation of the percentage recoveries were determined from the molecular weights of the cerebrosides. (Composition of the fatty acids and long-chain bases were analysed for this purpose.)

The calculated sugar percentages for the glycolipids used in this study were: cerebroside, 23.6%; sulphatide, 21.6%; monogalactosyl diglyceride, 21.6%; digalactosyl diglyceride, 37.6%; glucosyl ceramide, 22.0%; and lactosyl ceramide, 38.9%.

TABLE III

ESTIMATION OF GALACTOSE IN CEREBROSIDE SAMPLES

Values (after 6-h hydrolysis) are the means of duplicates.

μg/analysis	*Internal standard (arabinose) (μg)*	*Galactose*		*Recovery (%)*
		Found (μg)	*Calculated* (μg)*	
1840.0	174.5	403.4	434.3	92.8
1436.0	115.6	368.0	345.0	107.7
1335.0	115.6	336.4	315.1	106.6
1020.0	174.5	230.4	240.7	95.8
790.2	40.1	184.9	185.4	99.1
718.0	115.6	336.4	315.1	106.6
592.6	40.1	137.4	137.6	99.6
434.1	69.8	100.8	102.4	98.5
430.8	115.6	100.2	101.9	98.2
260.7	69.8	63.6	61.5	103.4

* Mean molecular weight was determined (see text).

TABLE IV

RECOVERY OF GALACTOSE FROM VARIOUS GLYCOLIPIDS

After 6-h hydrolysis.

Glycolipid	*Galactose (%)*	*n*
Cerebroside*	101.1 ± 5.2	11
Sulphatide*	97.8 ± 2.4	7
Monogalactosyl diglyceride**	97.5 ± 3.8	4
Digalactosyl diglyceride**	99.2 ± 3.3	4

* From brain.
** From plants.

DISCUSSION

Analysis of sugars as their alditol acetates in biological materials by GLC is well documented. The method originally based on the work of Gunner *et al.*[4] has been extended by Sawardeker *et al.*[16] and applied to glycoproteins[17,18] and, recently, to the glycolipid field[19,20]. As the alditol acetate derivatives of individual sugars give only one peak in GLC, the method promised to be less complicated in evaluation of chromatograms and would possibly allow more accurate quantitation (see Fig. 1). In contrast to other derivatives like the trimethylsilyl[21] and trifluoroacetyl[22] derivatives, the alditol acetate method involves the hydrolysis of the glycolipid with aqueous acid. We are unaware of a systematic study regarding the number of problems encountered in the aqueous acid hydrolysis of glycolipids. For example, no systematic and quantitative investigation of a series of individual well-defined ceramide hexosides

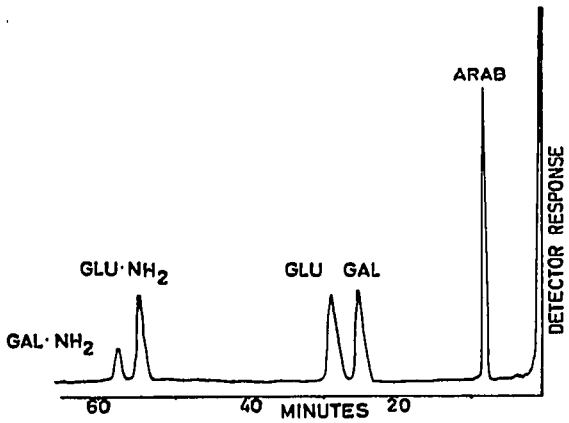

Fig. 1. Gas chromatogram of the alditol acetates of the standard monosaccharide mixture on a 1% OV-225 column. Conditions as in Table I.

by this procedure has appeared. The important aspects of the behaviour of various glycolipids in aqueous acid and the conditions necessary for quantitative cleavage of the sugar from the ceramide moiety are yet to be clarified, although scattered reports dealing with this problem are available in the literature[19,20]. It is, however, known that during the methanolic acid hydrolysis, the ceramide–glucose bond is more difficult to cleave than the ceramide–galactose bond[1,23]. We have now found that for the quantitative estimation of the sugars from ceramide glucosides the time of aqueous hydrolysis with 1 *N* hydrochloric acid has to be increased to 12 h at 100°

TABLE V

SUGAR ESTIMATION FROM CERAMIDE MONO- AND DIHEXOSIDES

Values (after 12-h hydrolysis) are the means of duplicates.

Glycolipid	*μg/analysis*	*Internal standard (arabinose) (μg)*	*Sugar*		*Recovery (%)*
			Found (μg)	*Calculated* (μg)*	
Galactosyl ceramide (Cer–Gal)	150.5	40.0	33.8	35.5	95.2
	225.8	40.0	53.4	53.3	100.3
	255.9	40.0	59.9	60.4	99.2
	331.1	40.0	74.1	78.1	94.9
Glucosyl ceramide (Cer–Glu)	75.0	40.0	15.1	16.5	91.9
	125.0	40.0	26.1	27.5	95.1
	175.0	40.0	39.2	38.5	101.8
	150.0	40.0	31.2	33.0	94.7
	250.0	40.0	53.6	54.9	97.5
Galactosyl glucosyl ceramide (Cer–Glu–Gal)	273.2	20.32	101.1	106.3	95.1
	426.8	20.32	166.1	166.0	100.0
	580.6	20.32	224.7	225.9	98.5
	751.3	20.32	301.6	292.3	103.2

* From the mean molecular weight (see text).

from the 6 h used for the hydrolysis of ceramide galactosides. (With a 6-h hydrolysis, the extent of the release of the sugar from Cer–Glu–Gal varies between 56 and 76%.) To know whether the galactosyl ceramide survives the longer period of hydrolysis a batch of galactosyl ceramide samples were also hydrolyzed for 12 h, like the ceramide glucosides and ceramide lactosides. Results (in Table V) show that the quantitative nature of the recovery from galactosyl cerebrosides remains unaltered with an increase in the time of hydrolysis up to 12 h and Cer–Glu and Cer–Glu–Gal give quantitative values under these conditions.

When dealing with ceramide polyhexosides, the galactose to glucose ratio is an important aspect which helps in knowing the structure of these substances. Fig. 2 shows the chromatograms of the various ceramide hexosides of the erythrocyte stroma which were analysed for their galactose to glucose ratio. The method has also been applied to examine the nature of the sugars in a case of lipidosis[14].

In order to investigate the possibility of a quantitative determination of the

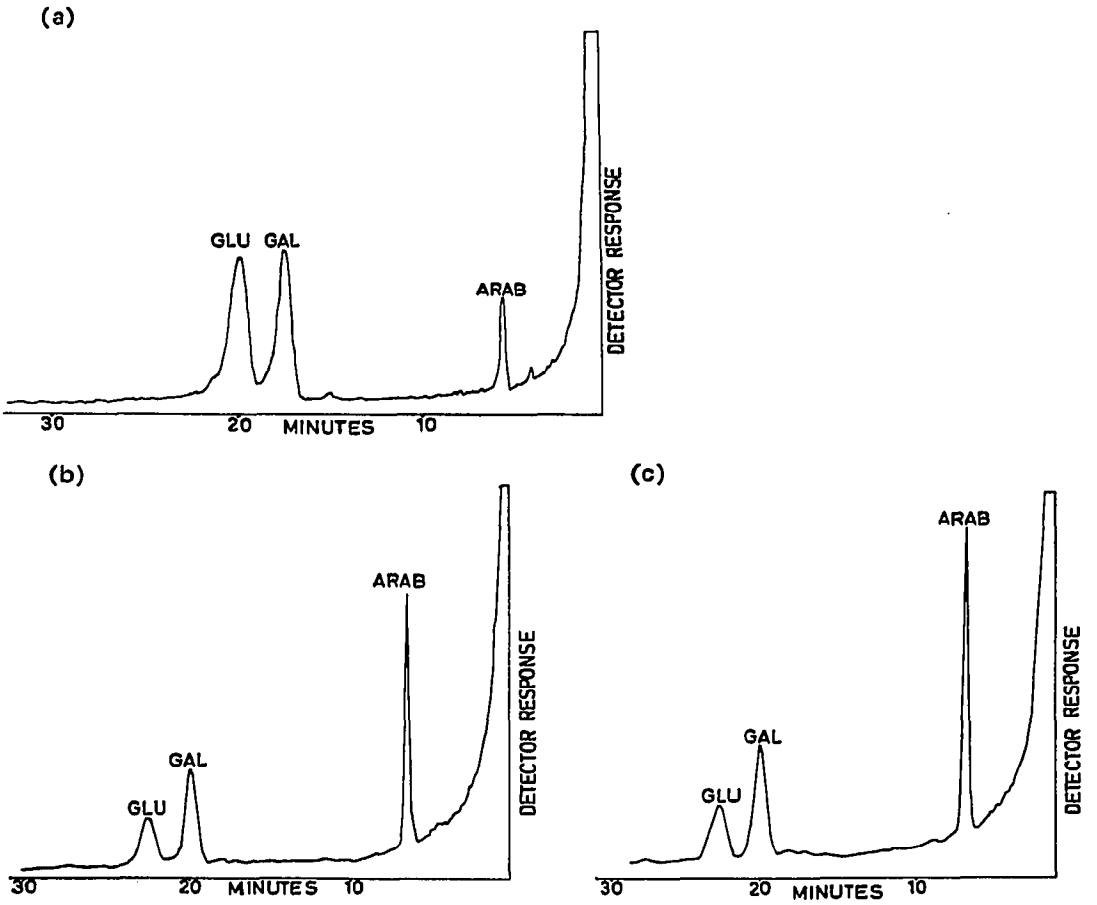

Fig. 2. Gas chromatograms of the sugars from the polyhexosides of human erythrocyte ghost lipids. (a) Cer–Glu–Gal: Gal to Glu ratio = 0.97; (b) Cer–Glu–Gal–Gal: Gal to Glu ratio = 2.07; (c) Cer–Glu–Gal–Gal–Gal–NHAc: Gal to Glu ratio = 1.92.

TABLE VI

ESTIMATION OF GALACTOSE IN LIPID MIXTURES

Values in parentheses are duplicates. Abbreviations: Cereb = cerebroside; PE = phosphatidylethanolamine fraction; PC = phosphatidylcholine fraction; Sulph = sulphatide; Sph = sphingomyelin.

Components	*Total weight* (*μg*)	*Glycolipid* (*μg*)	*Internal standard* (*arabinose*) (*μg*)	*Galactose*		*Recovery* (%)
				Found (*μg*)	*Expected* (*μg*)	
Cereb + PE + PC + Sph	1895.8	359.7	69.8	75.8 (77.1)	82.4	92.0 (93.6)
Cereb + PE + PC + Sph	1715.9	179.9	87.3	42.5 (42.2)	42.9	98.9 (98.4)
Sulph + Sph	1035.0	672.7	69.8	143.8 (145.3)	146.3	99.0 (99.3)
Sulph + Sph	690.0	483.0	69.8	96.7 (96.9)	101.7	95.9 (96.0)

neutral sugar directly from the Folch lower-phase lipids of the brain, we made synthetic mixtures of the major phospholipids present in brain lipids and galactolipids. Sugar analysis of artificial mixtures gave quantitative values for galactose (Table VI). Preliminary experiments with Folch[7] lower-phase lipids from some organs showed that inositol present in these lipids also gave a single peak with a higher retention time than that of glucose or galactose. This was confirmed with test samples of inositol and phosphatidylinositol. However, further studies are necessary to find out the quantitative nature of inositol and hexose estimations from total lipid extracts of various tissues. It should be mentioned here that the hydrolytic conditions used in this study are not sufficient for the quantitative estimation of inositol.

A comparison of this method with other gas chromatographic methods for neutral sugars shows that, unlike the other derivatives which produce four to five peaks for the O-methyl glycosides, the alditol acetates form single peaks in GLC, as mentioned earlier. In addition the acetate derivatives, unlike the others, do not decompose easily and because of less baseline drift in their analysis and better resolution are easy to quantitate. A report of an application of this method to gangliosides has recently appeared[24].

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REFERENCES

1 C. C. Sweeley and D. E. Vance, in G. V. Marinetti (Editor), *Lipid Chromatographic Analysis*, Vol. 1, Marcel Dekker, New York, 1967, p. 465.
2 C. C. Sweeley, R. Bentley, M. Makita and W. W. Wells, *J. Amer. Chem. Soc.*, 85 (1963) 2493.
3 J. R. Clamp, T. Bhatti and R. E. Chambers, *Methods Biochem. Anal.*, 19 (1971) 229.
4 S. W. Gunner, J. K. N. Jones and M. B. Perry, *Can. J.Chem.*, 39 (1961) 1892.
5 S. Abdel-Akher, J. H. Sloneker and A. Jeanes, *J. Amer. Chem. Soc.*, 73 (1951) 4691.

6 J. Metz, W. Ebert and H. Weicker, *Chromatographia*, 4 (1971) 345.
7 J. Folch, M. Lees and G. A. Sloane-Stanley, *J. Biol. Chem.*, 226 (1957) 496.
8 R. M. C. Dawson, *Biochem. J.*, 75 (1960) 45.
9 C. C. Sweeley, *J. Lipid Res.*, 4 (1963) 402.
10 H. Wagner, *Fette, Seifen, Anstrichm.*, 62 (1960) 1115.
11 A. Etzrodt and H. Debuch, *Hoppe-Seyler's Z. Physiol. Chem.*, 351 (1970) 603.
12 D. E. Vance and C. C. Sweeley, *J. Lipid Res.*, 8 (1967) 621.
13 K. Suzuki, *J. Neurochem.*, 18 (1971) 503.
14 R. Kannan, H. B. Tjiong, H. Debuch and H. R. Wiedemann, *Hoppe-Seyler's Z. Physiol. Chem.*, in press.
15 G. Tschöpe, *Hoppe-Seyler's Z. Physiol. Chem.*, 352 (1971) 71.
16 J. S. Sawardeker, J. A. Sloneker and J. Jeanes, *Anal. Chem.*, 37 (1965) 1602.
17 W. F. Lehnhardt and R. J. Winzler, *J. Chromatogr.*, 34 (1968) 471.
18 W. Ebert, J. Metz, H. Weicker and D. Roelcke, *Hoppe-Seyler's Z. Physiol. Chem.*, 352 (1971) 1309.
19 H. Yang and S. Hakomori, *J. Biol. Chem.*, 246 (1971) 1192.
20 D. B. Weinstein, J. B. Marsh, M. C. Glick and L. Warren, *J. Biol. Chem.*, 245 (1970) 3928.
21 C. C. Sweeley and B. Walker, *Anal. Chem.*, 36 (1964) 1461.
22 J. F. Zanetta, W. C. Breckenridge and G. Vincendon, *J. Chromatogr.*, 69 (1972) 291.
23 R. E. Chambers and J. R. Clamp, *Biochem. J.*, 125 (1971) 1009.
24 M. Holm, E. Mänsson, L. Varnier and L. Svennerholm, *Biochim. Biophys. Acta*, 280 (1972) 356.