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Note

Micro-scale separation of normal and hydroxy fatty acid methyl esters on a Florisil column

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Glycosphingolipid fatty acids are usually analysed by gas–liquid chromatography (GLC) following methanolysis with gaseous hydrogen chloride dissolved in dry methanol. Under appropriate conditions this procedure yields long-chain bases, methyl glycosides and fatty acid methyl esters. These esters are extracted from methanol–hydrogen chloride with repeated addition of *n*-hexane[1]. In many instances the mixture of fatty acid methyl esters cannot be analysed directly by GLC because of the presence of 2-hydroxy fatty acids. Normal and hydroxy fatty acid methyl esters are usually separated by thin-layer chromatography and extracted from the silica gel[2]. We propose here an alternative method involving the rapid and quantitative separation of normal and hydroxy fatty acid methyl esters on a small Florisil column. The recoveries and amounts of each class of esters were determined by GLC.

EXPERIMENTAL

Materials

Florisil (60–100 mesh) was obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.) and used without any treatment. All solvents were Nanograde materials from Mallinckrodt (St. Louis, Mo., U.S.A.). Recovery tests were performed with calibrated mixtures of fatty acid methyl esters bought from Applied Science Labs. (State College, Pa., U.S.A.). All tubes were acid washed, thoroughly rinsed with distilled water and oven dried. Pasteur pipettes were rinsed with *n*-hexane before use.

Methods

A 15-cm long pasteur pipette was placed in a 12-cm high test-tube filled with *n*-hexane. A disc, 5 mm in diameter, was punched out of a Whatman GF-B glass-fibre paper sheet and was carefully positioned just above the capillary section of the pipette. A 0.50-g amount of dry Florisil powder was poured into the pipette and the Florisil bed was gently suspended in *n*-hexane with a 23-cm long pasteur pipette in order to expel air bubbles. The column was then removed from the test-tube of *n*-hexane and washed with 5 ml of *n*-hexane–diethyl ether (95:5), the washings being discarded.

The sample, dissolved in 1 ml of *n*-hexane–diethyl ether, was immediately

loaded on the column and was successively eluted with 6 ml of *n*-hexane–diethyl ether and 6 ml of ethyl acetate. Normal fatty acid methyl esters were recovered in *n*-hexane–diethyl ether and hydroxy esters in ethyl acetate. This small Florisil column had a high flow-rate (2 ml/min). In order to prevent its drying between each elution step, solvents were pipetted into separate test-tubes before the run.

The efficiency of the separation was tested with mixtures of even-carbon-number fatty acid methyl esters. An aliquot of methyl heneicosanoate was added to each fraction eluted from the column and subsequently used as an external standard for quantitative determination.

Solvents were evaporated under nitrogen in a heating block at 40°. Normal esters were dissolved in *n*-hexane. Hydroxy esters were silylated, dried and re-dissolved in *n*-hexane prior to chromatography[3].

Analyses were performed on a Packard gas chromatograph equipped with a flame-ionization detector. Normal and hydroxy saturated fatty acid methyl esters were separated on a 6-ft. column packed with 3% OV-1 on Anachrom ABS with temperature programming from 175° to 310° at 5°/min. Saturated and unsaturated fatty acid methyl esters were analysed isothermally at 170° or 200°, depending on the chain length range of the fatty acids, on a 6-ft. column packed with 10% EGSS-X on Gas-Chrom P. Peak areas were determined with a Hewlett-Packard 3380A integrator.

RESULTS AND DISCUSSION

A Florisil column has already been used by Kishimoto and Radin[1] to separate normal and hydroxy fatty acid methyl esters on a preparative scale. The reduction of the column size and its elution with a similar solvent system gave unsatisfactory results: all esters, loaded in *n*-hexane on the column, remained adsorbed even after washing with 20 ml of *n*-hexane and they were eluted in *n*-hexane–diethyl ether (90:10) without separation.

Methyl esters of the major glycospingolipid fatty acids were used to find a suitable solvent system and to test column recoveries. *n*-Hexane–diethyl ether (95:5) allowed the elution of normal esters. Hydroxy esters, kept on the column in this first solvent, were eluted with ethyl acetate. A step-by-step procedure was followed in order to determine the optimal conditions for separation and elution. The sample, dissolved in 1 ml of *n*-hexane–diethyl ether (95:5), was loaded on the column and was eluted with a further 9 ml of the same solvent, which was collected in 3-ml fractions. Elution was continued with 6 ml of ethyl acetate, which was collected in 2-ml fractions (Table I). The major proportion of normal esters was recovered in the two first fractions; less than 0.5% of the initial load could be found in fraction 3. The hydroxy fatty acid methyl esters were eluted almost entirely with 4 ml of ethyl acetate but a small proportion (2–3%) left the column with *n*-hexane–diethyl ether. In order to keep cross-contaminations below 0.5%, the elution was performed with only 6 ml of *n*-hexane–diethyl ether followed by 6 ml of ethyl acetate.

The column was loaded with 100–300 μg of normal fatty acid methyl esters and 50–150 μg of hydroxy esters. Similar recoveries and extents of cross-contamination were observed when the column was loaded with 10 μg of normal and 5 μg of hydroxy fatty acid methyl esters.

The recovery of the esters from the column was nearly quantitative, as shown

TABLE I

RECOVERY OF FATTY ACID METHYL ESTERS FROM THE FLORISIL COLUMN IN SUCCESSIVE FRACTIONS OF ELUATE

Results are expressed as a percentage of the amount loaded on the column. Amounts of individual esters were determined by GLC with methyl heneicosanoate as external standard.

*Fatty acid methyl ester**	*Fraction No.*						*Total recovery (%)*
	1	*2*	*3*	*4*	*5*	*6*	
	Hexane–diethyl ether (95:5) (ml)			*Ethyl acetate (ml)*			
	1 + 3	*+3*	*+3*	*2*	*+2*	*+2*	
n 16:0	92.5	4.5	0.1				97.1
n 16:1	92.9	5.4					98.3
h 16:0		0.4	1.9	88.5	6.9	0.4	98.1
n 18:0	93.2	5.3	0.0				98.5
n 18:1	92.9	5.3	0.2				98.4
h 18:0		0.4	2.3	91.0	7.0	0.3	101.0
n 20:0	92.7	5.5	0.1				98.3
n 20:1	94.9	5.7	0.2				100.6
h 20:0		0.1	2.4	93.0	4.5	0.0	100.0
n 22:0	93.8	5.8	0.1				99.7
n 22:1	91.9	6.9	0.3				99.1
h 22:0		0.2	2.6	93.0	4.3	0.1	100.2
n 24:0	91.7	5.3	0.2				97.2
n 24:1	92.1	5.9	0.2				98.2
h 24:0		0.1	2.0	94.0	3.8	0.1	100.0

* n = normal; h = hydroxy; carbon chain length (*e.g.*, 16) followed by number of double bonds (*e.g.*, 1) after colon.

in Table I. However, losses of shorter chain fatty acid methyl esters were observed in some instances.

It was found that evaporation was a sensitive step. In order to investigate this aspect, a mixture of equal amounts of methyl esters was dried and left at 40° under a stream of nitrogen for various periods of time (Table II). After 3 min, 39% of methyl laurate and 8% of methyl myristate had been lost. After 17 min, 95% and 31% of

TABLE II

LOSSES OF FATTY ACID METHYL ESTERS DUE TO EVAPORATION AT 40° UNDER NITROGEN

A mixture of methyl esters (50 μg each) was kept dry at 40° under a stream of nitrogen for different periods up to 17 min. Peak areas resulting from GLC analyses are expressed as percentages of the 18:0 peak area taken as a reference.

*Fatty acid methyl ester**	*Heating times at 40° (min)*			
	0	*3*	*7*	*17*
n 12:0	96	61	36	5
n 14:0	99	92	86	69
n 16:0	100	99	98	96
n 18:0	100	100	100	100

* Abbreviations as in Table I.

these esters, respectively, were lost. Palmitic and palmitoleic acids, which are common components of glycosphingolipids, were lost to a lesser extent under these conditions but their recoveries were decreased on exposure to higher temperatures for shorter periods of time. In conclusion, care should be taken not to allow fatty acid methyl esters to dry under nitrogen for a longer period than necessary and not to use a temperature higher than 40°.

The Florisil column initially proposed by Kishimoto and Radin[1] for preparative purposes was adapted to the analytical separation of normal and hydroxy fatty acid methyl esters by reducing the column size and increasing the polarity of the solvents. The large volumes of solvent usually required in Florisil chromatography were avoided and the risk of sample contamination was decreased.

Previously two other methods have been proposed for the analytical separation of normal and hydroxy fatty acids: silicic acid column chromatography and thin-layer chromatography. Free fatty acids were separated by Kishimoto and Radin[4] on silicic acid columns but the flow-rates were so slow that air pressure was necessary in order to accelerate elution: this requirement, together with the use of benzene as solvent, made this method difficult and unsafe. Methyl esters of normal and hydroxy fatty acids were more easily separated by thin-layer chromatography[2] but it was often difficult to ensure their quantitative elution and to remove the silica gel.

The Florisil column built and used as described here was found to be easy to handle and did not require more than 15 min in order to achieve a good separation of the two classes of fatty acid methyl esters freed by glycosphingolipid methanolysis.

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